

m/H

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference L/VV37/1	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/EP 99/06302	International filing date (day/month/year) 27/08/1999	(Earliest) Priority Date (day/month/year) 27/08/1998
Applicant GENOCLIPP BIOTECHNOLOGY B.V. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.
 It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the International search was carried out on the basis of the International application in the language in which it was filed, unless otherwise indicated under this item.

the International search was carried out on the basis of a translation of the International application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the International application, the International search was carried out on the basis of the sequence listing :

contained in the International application in written form.

filed together with the International application in computer readable form.

furnished subsequently to this Authority in written form.

furnished subsequently to this Authority in computer readable form.

the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the International application as filed has been furnished.

the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. Certain claims were found unsearchable (See Box I).

3. Unity of invention is lacking (see Box II).

4. With regard to the title,

the text is approved as submitted by the applicant.

the text has been established by this Authority to read as follows:

5. With regard to the abstract,

the text is approved as submitted by the applicant.

the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

as suggested by the applicant.

because the applicant failed to suggest a figure.

because this figure better characterizes the invention.

None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No

CT/EP 99/06302

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	C12N15/60	C12N15/70	C12N15/82	C12N9/88	C12N5/10
	C12N1/19	C12N1/21	C12P5/00	C12P17/18	A01H5/00
	//C12R1:19, C12R1:84, C12R1:865				

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BRODELIUS P. ET AL.: "Metabolic engineering of plant secondary metabolism: a tool to improve the productivity of plant cell cultures?" ABSTRACT PAPERS OF THE AMERICAN CHEMICAL SOCIETY, 213 MEETING, April 1997 (1997-04), page AGFD026 XP002091772 abstract</p> <p>---</p>	1-42
A	<p>WOERDENBAG H J ET AL: "Progress in the research of artemisinin -related antimalarials: an update." PHARMACY WORLD AND SCIENCE, (1994 AUG 5) 16 (4) 169-80. REF: 157 JOURNAL CODE: B07. ISSN: 0928-1231., XP002091773 Netherlands the whole document</p> <p>---</p> <p style="text-align: right;">-/-</p>	1-42

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the International filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the International search

11 February 2000

Date of mailing of the International search report

28/02/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Kania, T

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/06302

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VAN GELDRE E. ET AL.: "State of the art of the production of the antimalarial compound artemisinin in plants" PLANT MOLECULAR BIOLOGY, vol. 33, no. 2, 1997, pages 199-209, XP002091774 the whole document ----	1-42
A	WALLAART T. ET AL.: "Bioconversion of dihydroartemisinic acid into the new antimalarial drug artemisinin" PHARMACY WORLD AND SCIENCE, vol. 16, no. 3, 1994, page C4 XP002091775 abstract	1-42
A	VERGAUWE A. ET AL.: "Agrobacterium tumefaciens-mediated transformation of <i>Artemisia annua</i> L. and regeneration of transgenic plants" PLANT CELL REPORTS, vol. 15, no. 12, 1996, pages 929-933, XP002091776 the whole document	1-42
A	WO 94 00584 A (WORCESTER POLYTECH INST) 6 January 1994 (1994-01-06) the whole document	1-42
A	MATSUSHITA Y ET AL: "Cloning and analysis of a cDNA encoding farnesyl diphosphate synthase from <i>Artemisia annua</i> " GENE, NL, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 172, no. 2, 26 June 1996 (1996-06-26), pages 207-209, XP004042738 ISSN: 0378-1119 the whole document	1-42
A	BRODELIUS, PETER E.: "Metabolic engineering of secondary metabolism in <i>vanilla planifolia</i> and <i>artemisia annua</i> ." BOOK OF ABSTRACTS, 211TH ACS NATIONAL MEETING, NEW ORLEANS, LA, MARCH 24-28 (1996), BIOT-002 PUBLISHER: AMERICAN CHEMICAL SOCIETY, WASHINGTON, D. C., XP002130362 abstract	1-42

	-/-	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/06302

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PARK C. ET AL.: "Expression, secretion, and processing of rice alpha-amylase in the yeast <i>Yarrowia lipolytica</i>" <i>JOURNAL OF BIOLOGICAL CHEMISTRY</i>, vol. 272, no. 11, 1997, pages 6876-6881, XP002130363 cited in the application the whole document</p> <p>---</p>	17-20, 31,35,36
A	<p>CHANG C. ET AL.: "Improvement of heterologous protein productivity using recombinant <i>Yarrowia lipolytica</i> and cyclic fed-batch process strategy" <i>BIOTECHNOLOGY AND BIOENGINEERING</i>, vol. 59, no. 3, 5 August 1998 (1998-08-05), pages 379-385, XP002130364 the whole document</p> <p>-----</p>	17-20, 31,35,36

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 99/06302

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9400584	A 06-01-1994	NONE	

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference L/VV37/1	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP99/06302	International filing date (day/month/year) 27/08/1999	Priority date (day/month/year) 27/08/1998
International Patent Classification (IPC) or national classification and IPC C12N15/60		
Applicant GENOCLIPP BIOTECHNOLOGY B.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I Basis of the report
- II Priority
- III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV Lack of unity of invention
- V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI Certain documents cited
- VII Certain defects in the international application
- VIII Certain observations on the international application

Date of submission of the demand 23/03/2000	Date of completion of this report 12.10.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Heckl, K Telephone No. +49 89 2399 8430



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP99/06302

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-23 as originally filed

Claims, No.:

1-42 as originally filed

Drawings, sheets:

1-18 as originally filed

2. The amendments have resulted in the cancellation of:

the description, pages:
 the claims, Nos.:
 the drawings, sheets:

3. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP99/06302

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-29,33-42
	No:	Claims 30-32
Inventive step (IS)	Yes:	Claims 1-29,33-42
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-42
	No:	Claims

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP99/06302

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement.

1. Reference is made to the following document.:

D1: BRODELIUS P. ET AL.: 'Metabolic engineering of plant secondary metabolism: a tool to improve the productivity of plant cell cultures?' ABSTRACT PAPERS OF THE AMERICAN CHEMICAL SOCIETY, 213 MEETING, April 1997 (1997-04), page AGFD026 XP002091772

2. Novelty (Art.33(2) PCT) of claims 30-32

It follows from the cited prior art (see the ISR and the description, pages 1-2) that the source of artemisinin of claims 30-32 also embraces naturally artemisinin producing sources. Therefore, these claims cannot be considered novel.

3. Novelty (Art.33(2) PCT) and Inventiveness (Art.33(3) PCT) of claims 1-29, 33-42

D1 formally discloses the increased production of artemisinin in cells of *Artemisia annua* by up-regulation of "a sesquiterpene cyclase" (introduction of extra copies of the cyclase gene).

The present application concerns the recombinant production of the antimalarial agent artemisinin in transgenic plants. The Applicants have elucidated the biosynthetic pathway leading from farnesyldiphosphate (FPP) to the end product artemisinin in *Artemisia annua*. An intermediate product is 4,11-amorphadiene (also called cadina-4,11-diene) which is the reaction product of the cyclization of FPP via the enzyme amorphadiene synthase (cyclase). The Applicants were successful in identifying this reaction to be the branch point in artemisinin synthesis, and isolating the enzyme and cloning the corresponding gene.

Regarding the teaching of D1 it is apparent that D1 does not contain any identification of the particular nature of the sesquiterpene cyclase which is to be upregulated in order to increase the production of artemisinin. It does also not

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP99/06302

contain any information how to obtain the same.

It is further noted that *A.annua* contains many sesquiterpene cyclases as has for example been illustrated by Bouwmeester et al. in *Phytochemistry* 52, 1999, 843-845 which document describes 14 different sesquiterpenes.

Taken together, D1 does neither disclose the teaching of the present claims 1-29 and 33-42 nor does it render obvious the same.

Accordingly, the subject-matter of claims 1-29 and 33-42 is novel and comprises an inventive step.

Re Item VIII

Certain observations on the international application

1. Claim 1 does not comprise any feature which allows to design a DNA sequence with the required properties. Accordingly, this claim attempts to define the subject-matter in terms of the result to be achieved. In this instance, however, such a formulation is not allowable because it is clearly possible to define the subject-matter in more concrete terms, viz. in terms of how the result is to be achieved (Art.6 PCT, lack of clarity).
2. Reference to an example introduced into the claims does not allow to identify the essential features of such a claim. Accordingly, claim 24 does not meet the requirements of Art.6 PCT (lack of clarity).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference L/VV37/1	FOR FURTHER ACTION		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP99/06302	International filing date (day/month/year) 27/08/1999	Priority date (day/month/year) 27/08/1998	
International Patent Classification (IPC) or national classification and IPC C12N15/60			
Applicant GENOCLIPP BIOTECHNOLOGY B.V. et al.			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I Basis of the report
- II Priority
- III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV Lack of unity of invention
- V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI Certain documents cited
- VII Certain defects in the international application
- VIII Certain observations on the international application

Date of submission of the demand 23/03/2000	Date of completion of this report 12.10.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Heckl, K Telephone No. +49 89 2399 8430



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP99/06302

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-23 as originally filed

Claims, No.:

1-42 as originally filed

Drawings, sheets:

1-18 as originally filed

2. The amendments have resulted in the cancellation of:

the description, pages:
 the claims, Nos.:
 the drawings, sheets:

3. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP99/06302

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-29,33-42
	No:	Claims 30-32
Inventive step (IS)	Yes:	Claims 1-29,33-42
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-42
	No:	Claims

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement.

1. Reference is made to the following document.:

D1: BRODELIUS P. ET AL.: 'Metabolic engineering of plant secondary metabolism: a tool to improve the productivity of plant cell cultures?' ABSTRACT PAPERS OF THE AMERICAN CHEMICAL SOCIETY, 213 MEETING, April 1997 (1997-04), page AGFD026 XP002091772

2. Novelty (Art.33(2) PCT) of claims 30-32

It follows from the cited prior art (see the ISR and the description, pages 1-2) that the source of artemisinin of claims 30-32 also embraces naturally artemisinin producing sources. Therefore, these claims cannot be considered novel.

3. Novelty (Art.33(2) PCT) and Inventiveness (Art.33(3) PCT) of claims 1-29, 33-42

D1 formally discloses the increased production of artemisinin in cells of *Artemisia annua* by up-regulation of "a sesquiterpene cyclase" (introduction of extra copies of the cyclase gene).

The present application concerns the recombinant production of the antimalarial agent artemisinin in transgenic plants. The Applicants have elucidated the biosynthetic pathway leading from farnesylidiphosphate (FPP) to the end product artemisinin in *Artemisia annua*. An intermediate product is 4,11-amorphadiene (also called cadina-4,11-diene) which is the reaction product of the cyclization of FPP via the enzyme amorphadiene synthase (cyclase). The Applicants were successful in identifying this reaction to be the branch point in artemisinin synthesis, and isolating the enzyme and cloning the corresponding gene.

Regarding the teaching of D1 it is apparent that D1 does not contain any identification of the particular nature of the sesquiterpene cyclase which is to be upregulated in order to increase the production of artemisinin. It does also not

contain any information how to obtain the same.

It is further noted that *A.annua* contains many sesquiterpene cyclases as has for example been illustrated by Bouwmeester et al. in *Phytochemistry* 52, 1999, 843-845 which document describes 14 different sesquiterpenes.

Taken together, D1 does neither disclose the teaching of the present claims 1-29 and 33-42 nor does it render obvious the same.

Accordingly, the subject-matter of claims 1-29 and 33-42 is novel and comprises an inventive step.

Re Item VIII

Certain observations on the international application

1. Claim 1 does not comprise any feature which allows to design a DNA sequence with the required properties. Accordingly, this claim attempts to define the subject-matter in terms of the result to be achieved. In this instance, however, such a formulation is not allowable because it is clearly possible to define the subject-matter in more concrete terms, viz. in terms of how the result is to be achieved (Art.6 PCT, lack of clarity).
2. Reference to an example introduced into the claims does not allow to identify the essential features of such a claim. Accordingly, claim 24 does not meet the requirements of Art.6 PCT (lack of clarity).

09/763822

Rec'd PCT/PTO 26 FEB 2001

ARNOLD SIEDSMA

ATTORNEYS AT LAW PATENT ATTORNEYS

ATTORNEYS AT LAW*

Jhr dr R.E.P.de Ranitz
Ms mr L.A.C. Beukers
Mr P.P.J.M.Verhaag
Mr M.A.A.van Wijngaarden

PATENT ATTORNEYS

Ir P.N. Hoorweg
Ir B.H.J.Schumann
Ir B.J.'t Jong
Dr ir H.W. Prins
Ir A.A.G. Land

Ir P. Eveleens Maarse
Ir E. Bartelds
Ms drs P.F.H.M.
van Someren
Ir H.Th. van den Heuvel
S. Duxbury B.Sc.
Ir J.A.M. Grootscholten
Ir J.P.E.Brants

Consultants
Ir L.M.C.J.Konings
Ir C.W. Bruin

Trademarks, Designs
Mr P.P.J.M.Verhaag
Ms L.J.Kraemer

European Patent Office
Patentlaan 2
2280 HV RIJSWIJK

P.O.Box 18558, NL-2502 EN THE HAGUE, 1 February 2001

Our ref.: L/VV37/ems/1

Your ref.: --

Re.: International Application No. PCT/EP(06302
in the name of: Genoclip Biotechnology B.V.

In the above identified case it was now found that one of the inventors and applicants for the United States was erroneously omitted upon filing the international application. It is requested pursuant to Rule 92^{bis} PCT to record the addition of the following person:

Hendrik Jan Bouwmeester
Kloosterkamp 14
6871 ZZ Renkum
Netherlands

nationality: Netherlands

as an inventor and applicant for the United States before expiry of the international phase.

E-MAIL
X.400:
C=nl;A=400net;
S=Arnold+Siedsma
Internet:
Arnold+Siedsma@
IPrights.unisource.nl

THE HAGUE*
Sweelinckplein 1
Tel. (070) 365 48 33
Fax (070) 345 21 40
Int. +31

BRUSSELS
Avenue
de la Faisanderie 39
Tel. (02) 375 2937
Fax (02) 375 0838
Int. +32

MUNICH
Isartorplatz 5
Tel.(089) 22 38 88
Fax (089) 29 26 95
Int. +49

ALICANTE
Explanada de España 2
Tel. (06) 52 02 291
Fax (06) 52 02 298
Int. +34

Offices
The Hague*
Brussels
Munich
Alicante
Breda
Enschede
Leeuwarden
Utrecht

09/763822

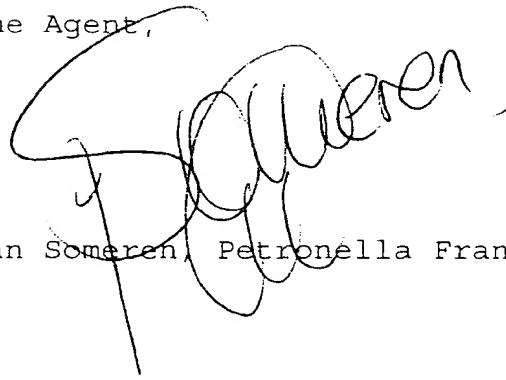
A R N O L D S I E D S M A

JC03 Rec'd PCT/PTC 26 FEB 2001

-2-

A Power of Attorney signed by Mr. Bouwmeester is enclosed.

The Agent,


Van Someren Petronella Francisca Hendrika Maria

Enclosures: 1

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING
OF A CHANGE(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)Date of mailing (day/month/year)
03 April 2001 (03.04.01)

From the INTERNATIONAL BUREAU

To:

VAN SOMEREN, Petronella,
Francisca, Hendrika, Maria
Arnold & Siedsma
Sweelinckplein 1
NL-2517 GK The Hague
PAYS-BASApplicant's or agent's file reference
L/VV37/1

IMPORTANT NOTIFICATION

International application No.
PCT/EP99/06302International filing date (day/month/year)
27 August 1999 (27.08.99)

1. The following indications appeared on record concerning:

 the applicant the inventor the agent the common representative

Name and Address

State of Nationality

State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

 the person the name the address the nationality the residence

Name and Address

BOUWMEESTER, Hendrik, Jan
Kloosterkamp 14
NL-6871 ZZ Renkum
Netherlands

State of Nationality

State of Residence

NL

NL

Telephone No.

Facsimile No.

Teleprinter No.

3. Further observations, if necessary:

Additional applicant/inventor for US only.

4. A copy of this notification has been sent to:

 the receiving Office the designated Offices concerned the International Searching Authority the elected Offices concerned the International Preliminary Examining Authority other:The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer

C. Cupello

Facsimile No.: (41-22) 740.14.35

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION
(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
 United States Patent and Trademark
 Office
 Box PCT
 Washington, D.C.20231
 ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 24 May 2000 (24.05.00)	Assistant Commissioner for Patents United States Patent and Trademark Office Box PCT Washington, D.C.20231 ETATS-UNIS D'AMERIQUE
International application No. PCT/EP99/06302	Applicant's or agent's file reference L/VV37/1
International filing date (day/month/year) 27 August 1999 (27.08.99)	Priority date (day/month/year) 27 August 1998 (27.08.98)
Applicant WALLAART, Thorvald, Eelco	

1. The designated Office is hereby notified of its election made:

in the demand filed with the International Preliminary Examining Authority on:

23 March 2000 (23.03.00)

in a notice effecting later election filed with the International Bureau on:

2. The election was
 was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer R. E. Stoffel Telephone No.: (41-22) 338.83.38
-----------------------------------------------------------------------------------------------------------------------------------	-------------------------------------------------------------------------



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/60, 15/70, 15/82, 9/88, 5/10, 1/19, 1/21, C12P 5/00, 17/18, A01H 5/00 // C12R 1:19, 1:84, 1:865		A2	(11) International Publication Number: WO 00/12725
			(43) International Publication Date: 9 March 2000 (09.03.00)
(21) International Application Number: PCT/EP99/06302		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 27 August 1999 (27.08.99)			
(30) Priority Data: 98202854.0 27 August 1998 (27.08.98) EP			
(71) Applicant (for all designated States except US): GENOCLIPP BIOTECHNOLOGY B.V. [NL/NL]; L.J. Zielstraweg 1, NL-9713 GX Groningen (NL).			
(72) Inventor; and			
(75) Inventor/Applicant (for US only): WALLAART, Thorvald, Eelco [NL/NL]; Duurswoldelaan 22, NL-9727 DJ Gronin- gen (NL).			
(74) Agent: VAN SOMEREN, Petronella, Francisca, Hendrika, Maria; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).			

(54) Title: TRANSGENIC AMORPHA-4,11-DIENE SYNTHESIS

(57) Abstract

The present invention relates to an isolated DNA sequence encoding a polypeptide having the biological activity of amorpho-4,11-diene synthase. This DNA sequence can be used for the transformation of bacteria, yeasts and plants for the production of amorpho-4,11-diene, a specific precursor in the synthesis of artemisinin, in the respective organisms. The invention also relates to these organisms.

TRANSGENIC AMORPHA-4,11-DIENE SYNTHESIS

The present invention relates to a DNA sequence, a polypeptide encoded by this sequence, and to the use of said DNA sequence and polypeptide in the production of amorphadiene.

5 Human malaria is a commonly occurring widespread infectious disease, caused in 85% of the cases by Plasmodium falciparum. This parasite is responsible for the most lethal form of malaria, malaria tropicana. Each year, malaria causes clinical illness, often very 10 severe, in over 100 million people of which eventually over 1 million individuals will die. Approximately 40% of the world's population is at risk of malaria infection (as estimated by the World Health Organization).

Malaria has traditionally been treated with 15 quinolines, such as quinine, chloroquine, mefloquine and primaquine, and with antifolates. Unfortunately, most P. falciparum strains have become resistant to chloroquine, and some have developed resistance to mefloquine and halofantrine as well. Thus, novel 20 antimarial drugs to which resistant parasites are sensitive are urgently needed. Artemisinin, as well as its semisynthetic derivatives are promising candidates here.

Artemisinin (Fig. 1), [3R-(3 α ,5a β ,6 β ,8a β ,
25 9 α ,12 β ,12aR*)]-Octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyran-4,3-j-1,2-benzodioxepin-10(3H)-one; molecular weight 282.35), also called arteannuin, qinghaosu or QHS, is a sesquiterpene lactone endoperoxide isolated from the aerial parts of the plant Artemisia annua L.

30 Artemisia annua L., also known as quinghao (Chinese), annual or sweet wormwood, or sweet annie is an annual herb native to Asia. A. annua, a member of the Asteraceae, belongs to the tribe Anthemideae of the Asteroideae, and is a large herb often reaching more than 35 2.0 m in height. It is usually single-stemmed with alternating branches. The aromatic leaves are deeply dissected and range from 2.5 to 5 cm in length. Artemisinin is mainly produced in the leaves as a

secondary metabolite at a concentration of 0.01 - 0.6% on a dry weight base in natural populations. Artemisinin is unique to the plant A.annua with one possible exception of A.apiacea L. The A.annua used in this invention is of 5 Vietnamese origin.

Because of its low concentration in plants, artemisinin is a relatively expensive resource for a drug. Current research has thus been aimed at producing artemisinin at a larger scale by organic synthesis. 10 However, because artemisinin consists of seven chiral carbon atoms, theoretically $2^7 = 128$ isomers can be formed of which only one is identical to artemisinin. Because of this complex structure of artemisinin, production of this compound by organic synthesis is not profitable from a 15 commercial point of view.

Genetic engineering of the biosynthetic pathway of artemisinin may give rise to higher artemisinin levels in plants. To be able to interfere in the biosynthesis of artemisinin, the biosynthetic pathway has to be known, 20 either completely or partially. Several attempts to elucidate the entire biosynthetic pathway have been undertaken. Until now, however, the exact pathway has remained largely unknown.

In the research that led to the present 25 invention, a unique pathway has been discovered which has not been published before. This pathway involves inter alia the formation of the artemisinin precursors amorpha-4,11-diene (1 β ,6 β ,7 β ,10 α H-amorpha-4,11-diene) and the hydroperoxide of dihydroartemisinic acid. These precursors 30 that were found in A.annua have not been described before in literature.

From literature it is known that terpene cyclases (synthases) are branch point enzymes, which likely play an important role in terpenoid biosynthesis. 35 The working hypothesis for this invention is thus that over-expression of such a branch point enzyme (terpene cyclase) may increase terpenoid production in an organism. Factors that may influence the success of such

an approach are, in the case of artemisinin, the number and nature of the subsequent biosynthetic steps leading to artemisinin. Fig. 2 shows the biosynthetic pathway of artemisinin as postulated by the present inventors.

5 This pathway is divided into three parts:

The first part (Part I) represents the terpenoid (Isoprenoid) pathway. This pathway is a general pathway. Farnesyl diphosphate (farnesyl pyrophosphate) (FPP), for example, is present in every living organism 10 and it is the precursor of a large number of primary and secondary metabolites. It has been established that FPP is the precursor of all sesquiterpenes. Thus, by definition FPP is the precursor of artemisinin.

Part II displays the cyclization of the general 15 precursor FPP into the highly specific precursor amorphadiene (also referred to as amorphadiene), the first specific precursor of artemisinin. In this pathway amorphadiene synthase is a branch point enzyme, having a key position in the biosynthetic pathway of artemisinin.

20 In part III, dihydroartannuic acid (DHAA), also called dihydroartemisinic acid, is photo-oxidatively converted into its hydroperoxide (DHAA-OOH). This hydroperoxide of DHAA will spontaneously oxidize into artemisinin. No enzymes are involved in this part of the 25 pathway and therefore it is impossible to alter artemisinin production by over-expression of genes involved in this part of the pathway.

Cytochrome P-450 catalyzed enzymes and an 30 enoate reductase are probably involved in the conversion of amorphadiene into DHAA, the transition state between part II and part III (see Fig. 3). Because no intermediates of this part of the pathway are known or present (accumulated) in detectable amounts, in the 35 plant, (except arteannuic acid, also called artemisinic acid or 4,11(13)-amorphadien-12-oic acid) it is likely that these precursors are very rapidly converted into DHAA. A rate limiting step in this part of the pathway is not very likely.

Taking all these aspects into account the inventors concluded that the most logical step to be altered by genetic interfering, is the conversion (cyclization) of FPP into amorphadiene by amorphadiene synthase.

The object of the present invention is therefore to provide a way in which artemisinin can be obtained via an at least partially biological route.

This object is achieved by the provision of a 10 DNA sequence which exhibits at least a 70% homology to the sequence as shown in Fig. 12, and which codes for a polypeptide having the biological activity of the enzyme amorphadiene synthase.

The biological activity of the enzyme 15 amorphadiene synthase relates to the conversion of the general precursor farnesyl pyrophosphate (FPP) into the specific artemisinin precursor amorpha-4,11-diene, which, in A.annua, is further converted to artemisinin. Suitable genes according to the invention can be selected by 20 testing the expression product of the gene for its ability to convert FPP into amorpha-4,11-diene.

By transforming a suitable host cell with the DNA sequence of the invention, the conversion of farnesyl pyrophosphate (FPP) into the highly specific precursor 25 amorphadiene can be increased or induced if this conversion route is not naturally present in the organism. In the latter case, the organism should comprise or be able to produce FPP. Suitable host cells are for example bacterial cells, such as E.coli, yeast 30 cells like Saccharomyces cerevisiae or Pichia pastoris and in particular oleaginous yeasts, like Yarrowia lipolytica, or plant cells such as those of A.annua.

Several plants are capable of producing large amounts of FPP making them potential organisms for 35 amorphadiene production.

The potential oleaginous yeast host cells, like, for example, Yarrowia lipolytica and Cryptococcus curvatus, have the capacity to accumulate up to about 50%

(dry weight) of storage carbohydrates in oil bodies, making them very interesting candidates as production organisms for large quantities of terpenes. According to the invention, a way to obtain high levels of terpene 5 accumulation is for example by means of re-direction of the metabolic flux in favor of the formation of amorpha-4,11-diene.

In analogy to the approach of an increased carotenoid production by the food yeast Candida utilis 10 through metabolic engineering of the isoprenoid pathway as done by Shimada et al. (Appl. Environ. Microbiol. **64**, 2676-2680 (1998)) the target genes according to the invention are acetyl CoA carboxylase (ACC, disruption), hydroxy-methyl-glutaryl CoA reductase (HMGR, 15 over-expression), and squalene synthase (SQS, disruption) to obtain an increase of the precursor supplies, and amorpha-4,11-diene synthase over-expression to obtain accumulation of amorphadiene in such yeast cells. Because several expression systems (for example Muller et al., 20 Yeast **14**, 1267-1283 (1998); Park et al., The Journal of Biological Chemistry **272**, 6876-6881 (1997); Tharaud et al., Gene **121**, 111-119 (1992)) and transformation systems (for example Chen et al., Appl. Microbiol. Biotechnol. **48**, 232-235 (1997)) are known for Y.lipolytica in 25 literature, transformation and expression of the previously mentioned target genes in Y.lipolytica is possible without serious technical problems.

By adding FPP to a culture medium further comprising the enzyme of the invention (isolated as 30 described in example 1), or transformed cells, e.g. E.coli, comprising the DNA sequence of the invention (as described in examples 3 and 4), which is expressed, FPP is converted into amorphadiene. Amorphadiene can then be used as a starting material for the production of 35 artemisinin.

Transformed cells in which amorphadiene is produced as a result of the expression of amorphadiene synthase of the invention can be used either in disrupted

form, by for example sonication, or as intact cells, as a source of amorphadiene.

Over-expression of the amorphadiene synthase encoding gene in A.annua will increase artemisinin 5 production, because the terpene cyclase is expected to be the rate limiting step.

The results of the present research (postulated biosynthetic pathway of artemisinin) make the presence of a single major rate limiting step at the place of the 10 amorphadiene synthase clear. Over-expression of the amorphadiene synthase encoding gene can increase the production of artemisinin in A.annua.

The chemical structure of the first specific precursor of artemisinin, a cyclization product of FPP, 15 was not known in literature. Neither has anyone so far detected such a compound in A.annua. Nevertheless it was possible to predict a likely structure for this cyclization product, based on the structure of DHAA and arteannuic acid (Fig. 3). The structure predicted in this 20 way was consistent with a compound which is known in literature as 4,11-amorphadiene (J.D. Connolly & R.A. Hill in: Dictionary of terpenoids, Chapman and Hill, London, England), as depicted in Fig. 4. This compound, isolated from Viguiera oblongifolia, has previously been 25 described by Bohlmann et al. under the incorrect name cadina-4,11-diene (Phytochemistry 23(5) 1183-1184 (1984)). Starting from arteannuic acid (isolated from A.annua), it was possible to synthesize amorphadiene. Amorphadiene obtained in this way was in all chemical and 30 physical aspects identical to amorphadiene as described by Bohlmann et al., and this standard was used to show the presence of amorphadiene in a terpene extract of A.annua.

A further object of the present invention is to 35 provide a polypeptide having the biological activity of the enzyme amorphadiene synthase, obtainable by a process as described in example 1. This polypeptide can be used to convert FPP into amorphadiene which subsequently can

be converted into artemisinin. Conversion can take place either in planta, when the polypeptide amorphadiene synthase is expressed in a plant that contains the necessary enzymes to further convert amorphadiene into 5 artemisinin, or in vitro when FPP and the polypeptide (either in isolated form or as an expression product in a cell) are brought together in an incubation mixture.

Amorphadiene, produced by a suitable host organism transformed with the DNA sequence of the 10 invention as precursor, can subsequently be chemically converted to dihydroarteannic acid. Dihydroarteannic acid per se can be used or in the production of artemisinin.

The chemical conversion of amorphadiene into 15 dihydroarteannic acid (Fig. 15) starts with the enantio-, stereo- and regioselective (anti-markownikoff) hydroboration of amorphadiene with BH_3 , yielding a trialkylborane, followed by an oxidation of the trialkylborane with $\text{NaOH}/\text{H}_2\text{O}_2$, yielding the alcohol 20 (Advanced Organic Chemistry, Jerry March, 4th Edition, Wiley, 1992). A mild oxidation of the alcohol to the acid can be obtained by PDC (pyridinium dichromate) without attacking the second double bond (Fig. 15) (Organic Synthesis, M.B. Smith, 1st Edition, McGraw-Hill, 1994).

25 Many genes encoding enzymes involved in the biosynthetic pathway of farnesyl diphosphate are cloned and known in literature. For A.annua, for example, the sequence of the farnesyl diphosphate synthase encoding gene is known in literature (Y. Matsushita, W-K. Kang and 30 V. Charlwood Gene, 172 (1996) 207-209). A further approach to introduce or increase the amorphadiene production in an organism, is to transform such an organism (for example A.annua) simultaneously with the DNA sequence of the invention with one or more genes 35 involved in the biosynthesis of farnesyl diphosphate. The expression of a fusion protein of amorphadiene synthase and farnesyl diphosphate synthase may be an example here.

(Sesqui)terpenes, such as amorphadiene, are also known as flavor and fragrance compounds in the food and perfume industry. In addition, terpenes play a role in plant-insect interactions, such as the attraction or 5 repulsion of insects by plants. Furthermore, dihydro-arteannuic acid, which is an intermediate in the metabolic route from amorphadiene into artemisinin in A.annua, can be used as an antioxidant.

Amorphadiene, obtained by (over)expression of 10 the DNA sequence of the invention, or by using the polypeptide (amorphadiene synthase) of the invention, can be applied for these purposes as well.

The plants that can be used for this invention are preferably plants already producing artemisinin. A 15 prime example is Artemisia annua, as this species contains the remainder of the pathway leading to artemisinin. However, this invention may also be used for the production of amorphadiene in plants, which, as mentioned before, can be used as a flavor or fragrance 20 compound or biocide, or can be converted to artemisinin, either chemically or by bioconversion using microorganisms, yeasts or plant cells.

The plant that can be used for the production of amorphadiene is preferably a plant already producing 25 sesquiterpenes, as these plants already have the basic pathway and storage compartments available, or a plant in which the biosynthesis of sesquiterpenoids can be induced by elicitation. The methods of this invention are readily applicable via conventional techniques to numerous plant 30 species, including for example species from the genera Carum, Cichorium, Daucus, Juniperus, Chamomilla, Lactuca, Pogostemon and Vetiveria, and species of the inducible (by elicitation) sesquiterpenoid phytoalexin producing genera Capsicum, Gossypium, Lycopersicon, Nicotiana, 35 Phleum, Solanum and Ulmus. However, also common agricultural crops like soybean, sunflower and rapeseed are interesting candidates here.

The invention will be further illustrated by the following examples, but will not be limited thereto. In the examples reference is made to the following figures:

5 **Fig. 1:** Structural formula of artemisinin.

Fig. 2: Postulated biosynthetic pathway of artemisinin in A. annua.

Fig. 3: Transition between part II and III of

Fig. 2: hypothetical conversion of amorphadiene into

10 dihydroartemisinic acid in A. annua.

Fig. 4: Structural formula of amorpha-4,11-diene.

Fig. 5: Radio-GC chromatograms of the [³H]-FPP assays. A. Flame Ionization Detector (FID) signal of 15 amorphadiene (reference). B. Radio signals of the ³H labeled assay products amorphadiene (retention time 14 min.) and farnesol (as a product of aspecific phosphohydrolase activity, retention time 28 min.) obtained with crude enzyme extract. C. Radio signal of 20 the ³H labeled assay product amorphadiene obtained with Mono-Q purified enzyme extract.

Fig. 6: Mass spectrum of reference amorphadiene compared with mass spectrum of the FPP assay with terpene cyclases (synthases) purified from A. annua. This 25 comparison yielded a quality score of 99%, corresponding with a maximum score of identicalness.

Fig. 7: Probe generated by PCR and cloned into pGEM 7Zf⁺.

Fig. 8: Nucleotide sequence and deduced amino 30 acid sequence of the probe (538 bp) generated by PCR with primers A and B.

Fig. 9: Released plasmid of a positive clone isolated from the cDNA library of induced A. annua.

Fig. 10: Nucleotide sequence and deduced amino 35 acid sequence of a positive clone (amorphadiene synthase encoding gene) isolated from the cDNA library of induced A. annua. The sequence is flanked with EcoRI (NotI) adapters (Gibco BRL).

Fig. 11: Part, between start and stop codon (flanked by NcoI and BamHI sites, respectively), of the amorphadiene synthase encoding gene cloned in the NcoI/BamHI site of the expression vector pET 11d.

5 **Fig. 12:** Nucleotide sequence and deduced amino acid sequence of the amorphadiene synthase encoding gene, between start and stop codon (flanked by NcoI and BamHI sites, respectively), obtained by PCR with primers C and D.

10 **Fig. 13:** SDS-PAGE gel: lanes 1 and 2 show pellet and supernatant of pET 11d, respectively (negative control); lanes 3 and 4 show pellet and supernatant of tobacco 5-epi-aristolochene synthase (TEAS) gene in pET 11d (positive control), lanes 5, 7, 9 and 6, 8, 10, 15 respectively show pellet and supernatant of amorphadiene synthase in pET 11d. All constructs were expressed in E.coli BL21 (DE3). The lanes with the pellet fractions of TEAS in pET 11d (positive controls) and amorphadiene synthase in pET 11d show a clear spot which was not 20 present in the negative control pET 11d. Mw is low Molecular Weight marker (Pharmacia Biotech).

25 **Fig. 14:** A. Flame Ionization Detector (FID) signals of amorpha-4,11-diene and farnesol (references); B. Radio-GC chromatograms of the [³H]-FPP-assays with intact BL21 (DE3) cells, transformed with the amorphadiene synthase encoding gene in the expression vector pET 11d; C. Radio-GC chromatograms of the [³H]-FPP-assays with the supernatant of sonicated BL21 (DE3) cells, transformed with the amorphadiene synthase 30 encoding gene in the expression vector pET 11d.

35 **Fig. 15:** Hypothetical chemical synthesis of dihydroarteannuic acid using amorpha-4,11-diene as a precursor. The reaction consists of an enantio-, stereo- and region selective (anti-markownikoff) hydroboration of amorphadiene with BH₃, followed by an oxidation of the formed trialkylboranes with NaOH/H₂O₂, yielding the alcohol. A mild oxidation of the alcohol to the acid can

be obtained with PDC (pyridinium dichromate) without attacking the second double bond.

Fig. 16: Determination of the molecular weight of amorpha-4,11-diene synthase by size-exclusion chromatography (gel filtration). -*- is activity curve; -▲- is molecular weight markers; — is molecular weight calibration line.

10 **EXAMPLES**

EXAMPLE 1

Conversion of farnesyl pyrophosphate into amorphadiene by amorphadiene synthase

A. Isolation, partial purification and identification of 15 amorphadiene synthase from A.annua

During enzyme isolation and preparation of the assays, all operations were carried out on ice or at 4°C. Ten grams of frozen young leaves from greenhouse-grown A.annua were ground in a pre-chilled mortar and pestle in 20 40 ml of pre-chilled buffer containing 25 mM MES (pH 5.5), 20% (v/v) glycerol, 25 mM sodium ascorbate, 25 mM NaHSO₃, 10 mM MgCl₂, and 5 mM DTT (buffer A) slurried with 1 g polyvinylpolypyrrolidone (PVPP) and a spatula tip of purified sea sand. Ten grams of polystyrene resin 25 (Amberlite XAD-4, Serva) were added and the slurry was stirred carefully for 10 min and then filtered through cheesecloth. The filtrate was centrifuged at 20,000g for 20 min (pellet discarded), and then at 100,000g for 90 min. A 3-ml subsample of the supernatant was desalted to 30 a buffer containing 15 mM MOPS (pH 7.0), 10% (v/v) glycerol, 1 mM sodium ascorbate, 10 mM MgCl₂, and 2 mM DTT (buffer B) and used for enzyme assays/product identification (see below at 'B').

The remainder of the supernatant was added to 35 12.5 g DEAE anion exchanger (Whatman DE-52), which had been rinsed several times with buffer A, and stirred carefully for 10 min. After centrifugation at 18,000g for 20 min, the supernatant was decanted and the DE-52 pellet

discarded. Proteins in the supernatant were precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 70%, careful stirring for 30 min, and centrifugation at 20,000g for 10 min. The resulting pellet was resuspended 5 in 6 ml buffer A and desalted to buffer B. After addition of glycerol up to 30% (v/v) this enzyme preparation could be frozen in liquid N_2 and stored at -80°C without loss of activity. 0.5 ml of this enzyme preparation was applied to a Mono-Q FPLC column (HR5/5, Pharmacia Biotech), 10 previously equilibrated with buffer B without sodium ascorbate, with 0.1% Tween-20. The enzyme was eluted with a gradient of 0-2.0 M KCl in the same buffer. For determination of enzyme activities, 50 μl of the 0.75-ml fractions were diluted 2-fold in an Eppendorf tube with 15 buffer B and 20 μM [^3H]FPP was added. The reaction mixture was overlaid with 1 ml of hexane to trap volatile products and the contents mixed. After incubation for 30 min at 30°C, the vials were vigorously mixed, and centrifuged briefly to separate phases. A portion of the 20 hexane phase (750 μl) was transferred to a new Eppendorf tube containing 40 mg of silica gel (0.035-0.07 mm, pore diameter 6 nm, Janssen Chimica) to bind terpenols produced by phosphohydrolases, and, after mixing and centrifugation, 500 μl of the hexane layer was removed 25 for liquid scintillation counting in 4.5 ml of Ultima Gold cocktail (Packard). The active fractions were combined, and an assay carried out to determine product identity (see below). After the Mono-Q step, the enzyme was separated from all other FPP-converting activities 30 (Fig. 5C). This enzyme preparation was used for the measurement of enzyme characteristics such as molecular weight and K_m . The molecular weight was determined using size-exclusion chromatography. 200 μl of the Mono-Q eluent was loaded on a Superdex 75 (H/R10/30, Pharmacia 35 Biotech) and eluted in the same buffer as used for Mono-Q. Enzyme activities in 0.5 ml fractions were determined as described for Mono-Q, but using undiluted eluent. The column was calibrated using cytochrome C, ribonuclease A,

α -chymotrypsinogen, ovalbumin and BSA (all from Sigma). The estimated molecular weight was 56 kDa (Fig. 16). Enzyme-kinetics were determined using 5- and 10-fold diluted Mono-Q eluted enzyme preparation and [3 H]-FPP 5 concentrations ranging from 0.25-100 μ M. K_m for amorphadiene synthase was 0.6 μ M.

B. Determination of product identity

For determination of product identity, 20 μ M [math>^3H]-FPP (Amersham; for radio-GC analysis) or 50 μ M unlabelled FPP (Sigma; for GC-MS analysis) were added to 10 1 ml of the enzyme preparations. After the addition of a 1 ml redistilled pentane overlay to trap volatile products, the tubes were carefully mixed and incubated 15 for 1 h at 30°C. Boiled samples were used as controls. Following the assay, the tubes were vigorously mixed. The 20 organic layer was removed and passed over a short column of aluminum oxide overlaid with anhydrous MgSO₄. The assay was extracted with another 1 ml of diethyl ether which was also passed over the aluminum oxide column, and the 25 column washed with 1.5 ml of diethyl-ether. For GC-analysis, the combined pentane/diethyl-ether mixture was slowly concentrated under a stream of N₂.

Radio-GLC was performed on a Carlo-Erba 4160 30 Series gas chromatograph equipped with a RAGA-90 radioactivity detector (Raytest, Straubenhardt, Germany). Sample components eluting from the column were quantitatively reduced before radioactivity measurement by passage through a conversion reactor filled with 35 platinum chips at 800°C. Samples of 1 μ l were injected in the cold on-column mode. The column was a fused silica capillary (30 m x 0.32 mm i.d.) coated with a film of 0.25 μ m of polyethylene glycol (EconoCap EC-WAX, Alltech Associates) and operated with a He-flow of 1.2 ml min⁻¹. The oven temperature was programmed to 70°C for 5 min, followed by a ramp of 5° min⁻¹ to 210°C and a final time 35 of 5 min. To determine retention times and peak identities (by co-elution of radioactivity with reference

standards), about 20% of the column effluent was split with an adjustable splitter to an FID (temperature 270°C). The remainder was directed to the conversion reactor and radio detector. H₂ was added prior to the 5 reactor at 3 ml min⁻¹, and CH₄ as a quench gas prior to the radioactivity detector (5 ml counting tube) to give a total flow of 36 ml min⁻¹. The major [³H]-labeled product co-eluted with the amorphadiene reference standard (retention time 14 min) (Fig. 5B). The second 10 radiolabeled product is farnesol, the product of aspecific phosphohydrolase activity. After the Mono-Q step, the enzyme was separated from all other FPP-converting activities (Fig. 5C). This enzyme preparation was used for the measurement of enzyme characteristics 15 such as molecular weight and K_m.

GC-MS analysis was performed using a HP 5890 series II GC and HP 5972A Mass Selective Detector (Hewlett-Packard) equipped with an HP-5MS or HP-Innowax column (both 30 m x 0.25 mm i.d., 0.25 μm df). The oven 20 was programmed at an initial temperature of 70°C for 1 min, with a ramp of 5°C min⁻¹ to 210°C and final time of 5 min. The injection port (splitless mode), interface and MS source temperatures were 175, 290 and 180°C, respectively, and the He inlet pressure was controlled by 25 electronic pressure control to achieve a constant column flow of 1.0 ml min⁻¹. Ionization potential was set at 70 eV, and scanning was performed from 30-250 amu. The (NH₄)₂SO₄ precipitated enzyme preparation was free of endogenous sesquiterpenes. GC-MS analysis on the two 30 different GC-columns of sesquiterpene products generated from FPP by this enzyme preparation showed that the main product had a mass spectrum and retention time equal to that of the semi-synthetically produced amorphadiene (Fig. 6).

EXAMPLE 2Isolation and characterization of the amorphadiene synthase encoding gene

A. Induction of transcription

5 As revealed in part III of Fig. 2, DHAA is photo-oxidatively converted into DHAA-OOH. In this reaction a reactive form of oxygen (singlet O₂) is added to DHAA. DHAA plays the role of an anti-oxidant, a scavenger of reactive oxygen species. Artemisinin is the 10 stable end product of this reaction in which reactive oxygen is stored. Under stress conditions, (for example photo-stress, frost, drought or mechanical damage) reactive species of oxygen are formed in the plant. In response to this reactive oxygen generally plants are 15 producing anti-oxidants. It is likely that A.annua will produce DHAA as anti-oxidant in response to this release of reactive oxygen. By exposing A.annua to stress conditions the transcription of the gene encoding amorphadiene synthase will be induced. To achieve this 20 situation A.annua plants grown under climate room conditions (23°C, 90% moisture, 3000 lux) were exposed to stress conditions by putting them for one hour at approximately 30% moisture (drought stress) and 6000 lux (photo stress) at 30°C.

25

B. Isolation of total RNA

Total RNA of stress induced plants (according to example 2.A) was isolated from young leaves by the method of Verwoerd et al. (Nucleic Acids Research 17(6), 30 2362 (1989)). DNase I (Deoxyribonuclease I, RNase free) was used to remove DNA from the RNA isolate. The DNase I was inactivated by exposure at 70°C during 15 minutes.

C. cDNA synthesis

35 The reverse transcription reaction was carried out in a 20 µl reaction containing 5 µg total RNA, 0.2 µg oligo (dT)₁₂, 0.5 mM each dATP, dTTP, dCTP and dGTP, 10 mM DTT, 2 U ribonuclease inhibitor (Gibco BRL), first strand

synthesis buffer (Promega) and catalyzed with 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase RNase H minus (Promega). After 1 h incubation at 37°C the reaction was stopped by storing 5 the reaction mixture at -20°C.

D. PCR-based probe generation

Based on comparison of sequences of terpenoid synthases, two degenerated primers were designed for two 10 conserved regions. The sequence of the sense primer (primer A) was 5'-GA(C/T) GA(G/A) AA(C/T) GGI AA(G/A) TT(C/T) AA(G/A) GA-3' and the sequence of the anti sense primer (primer B) was 5'-CC (G/A)TA IGC (G/A)TC (G/A)AA IGT (G/A)TC (G/A)TC-3'. PCR was performed in a total 15 volume of 100 μ l containing 0.5 μ M of each of these two primers, 0.2 mM each dNTP, 1 U Super Taq polymerase / 1x PCR buffer (HT Biotechnology LTD, Cambridge, England) and 2 μ l cDNA. The reaction was incubated in a thermocycler (PTC 150, MJ-research) with 1 minute denaturation at 20 95°C, 1 minute annealing at 40°C and 1 minute and 15 seconds elongation at 72°C during 40 cycles. Agarose gel electrophoresis revealed a single specific PCR product of approximately 550 bp (538 bp). Such a specific 25 amplification product was only obtained when using cDNA made of RNA isolated from stress induced plants. The PCR product was made blunt by using DNA polymerase I large fragment (Klenow), gel-purified and subcloned in Sma I digested pGEM 7zf(+) (Stratagene) (Fig. 7) and E.coli DH5 α (Gibco BRL) was transformed with this construct. The 30 inserts of 8 individual transformants were sequenced and they all had the same sequence as shown in Fig. 8.

E. cDNA Library construction

Synthesis of the second strand of the cDNA was 35 done analogous to the RiboClone® cDNA synthesis System (Promega). After ligation with EcoR I (Not I) adapters (Gibco BRL) with sequence:

5' - pGTCGACGCGGCCGCG-3'
3' - CAGCTGCGCCGGCGCTTAA-OH-5'

the double stranded DNA was ligated into λ ExCell
5 EcoRI/CIP (Pharmacia Biotech). For packaging and plating
of the cDNA library, the Ready-To-Go[®] Lambda Packaging
Kit (Pharmacia Biotech) was used. The titer of the
unamplified library was 1.2×10^6 plaque forming units.

10 F. Library screening

For library screening 200 ng of the PCR
amplified probe (Fig. 8) was gel purified, randomly
labeled with [α -³²P]dCTP, according to the manufacturer's
recommendation (Random Primed DNA Labeling Kit,
15 Boehringer Mannheim Biochemica) and used to screen
replica filters of 10^4 plaques of the cDNA library plated
on E.coli NM 522. The hybridization was performed for 16
h at 68°C in 1 M NaCl, 1% SDS and 10% PEG (5000-7000).
Filters were washed two times for 10 minutes at 50°C in 2
20 x SSC with 0.1% SDS and exposed for 16 h to a Fuji X-ray
film at -70°C. Clones yielding positive signals were
isolated through a second and third round of
hybridization. By transfecting E.coli NP66 (Pharmacia
Biotech) with the positive clones, plasmid releases (Fig.
25 9) were obtained according to the manufacturer's
instructions (Pharmacia Biotech). Sequencing of these
positive clones yielded a sequence as revealed in Fig.
10.

30 EXAMPLE 3

Expression of the amorphadiene synthase encoding gene in
E.coli BL21(DE3)

For functional expression the cDNA clone was
subcloned in frame into the expression vector pET 11d
35 (Stratagene). To introduce suitable restriction sites for
subcloning, the gene was amplified by PCR using a sense
primer (primer C) 5'-GTCGACAAACCCATGGCACTTACAGAA G-3'

(introducing a NcoI site at the start codon **ATG**) and an anti-sense primer (primer D):

5' -GGATGGATCCTCATATACTCATAGGATAAACG-3' (introducing a BamHI site directly behind the stop codon **TGA**). The PCR reaction was performed under standard conditions. After digestion with BamHI and NcoI, the PCR product (Fig. 12) and the expression vector pET 11d were gel purified and ligated together to yield a construct as revealed in Fig. 11.

10 To obtain expression, this gene construct (Fig. 11), pET 11d without an insert as negative control, and pET 11d with the tobacco 5-epi-aristolochene synthase (TEAS) gene (Back et al., Archives of Biochemistry and Biophysics 315(2) 527-532 (1994); Facchini & Chappell, 15 Proc. Natl. Acad. Sci. USA 89, 11088-11092 (1992); Back & Chappell, The Journal of Biological Chemistry 270, 7375-7381 (1995)) as positive control were transformed to E.coli BL21(DE3) (Stratagene), and grown overnight on LB agar plates supplemented with ampicillin at 37°C.

20 Cultures of 50 ml LB medium supplemented with ampicillin (100 µg/ml) and 0.25 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) were inoculated with these overnight cultures to $A_{600} = 0.5$ and grown for 3 h at 27°C. The cells were harvested by centrifugation during 8 minutes 25 at 2000 g and resuspended in 2 ml assay buffer. An aliquot of 1 ml resuspended cells was sonicated on ice four times for 5 seconds with 30 second intervals, centrifuged for 5 minutes at 4°C in a microfuge (13.000 rpm) and the supernatant used for cyclase enzyme activity 30 determinations and SDS-PAGE gel electrophoresis.

Expression of the amorphadiene synthase gene-pET 11d construct (Fig. 11) in E.coli BL21(DE3) yielded a protein of approximately 50 to 60 kDa as shown in Fig. 13 lane 5 to 10. This agrees well to the size of 35 amorphadiene synthase isolated from A.annua, which was determined to be 56 kDa (Fig. 16).

EXAMPLE 4Conversion of FPP into amorphadiene by amorphadiene synthase expressed in E.coli.

Besides the supernatant of sonicated cells, 5 also intact cells were used in the FPP assay. The FPP assay, GC-RAGA and GC-MS analyses were performed as described previously. Figs. 14 and 14A are revealing the GC-RAGA chromatograms of the assays with intact 10 transformed cells and with the supernatant of sonicated transformed cells, respectively. In both assays amorphadiene was produced. Identification of these assay products with the GC-MS gave a mass-spectrum identical to the mass-spectrum of the reference amorphadiene with a quality score of 99% (maximum score), mass spectra were 15 identical to the spectra as shown in Fig. 6. No amorphadiene was found in assays done with the positive and negative controls.

EXAMPLE 520 Expression of amorpha-4,11-diene synthase in transgenic tobacco

There are many ways to introduce DNA into a plant cell. Suitable methods by which DNA can be introduced into the plant cell include Agrobacterium 25 infection or direct delivery of DNA such as, for example, by PEG-mediated transformation of protoplasts (Omirulleh et al., Plant Molecular Biology 21, 415-428 (1993)) or electroporation, by acceleration of DNA coated microprojectiles (for example, microprojectile 30 bombardment) microinjection, etc.

Because Agrobacterium tumefaciens-mediated transformation of Artemisia annua and Nicotiana tabacum with a sesquiterpene cyclase gene is known in literature (Vergauwe et al., Plant Cell Reports 15, 929-933 (1996); 35 Hohn and Ohlrogge, Plant Physiol. 97, 460-462 (1991)) delivery of expression units (cassettes), containing the amorphadiene synthase encoding gene, mediated by Agrobacterium seemed to be a rational approach.

There are several binary vector systems suitable to transfer the amorphadiene synthase encoding gene assembled in an expression cassette behind a suitable promoter (for example, the cauliflower mosaic virus (CaMV) 35S promoter) and upstream of a suitable terminator (for example, the nopaline synthase transcription terminator (nos-tail)) to tobacco and/or A. annua.

Analogous to EXAMPLE 3, suitable restriction sites for subcloning were introduced by using PCR with a sense primer (primer G) 5'-GA GGA TCC ATG TCA CTT ACA GAA-3' introducing a BamHI site preceding the start codon ATG) and an anti-sense primer (primer H) 5'-AT GGA TCC TCA TAT ACT CAT AGG A-3' (introducing a BamHI site directly behind the stop codon TGA). After digestion with BamHI the PCR product and the plant-expression cassette pLV399 were gel purified and ligated to provide the gene encoding amorpha-4,11-diene synthase with the cauliflower mosaic virus 35S promoter and a nopaline synthase transcription terminator. The plant-expression cassette pLV399 is a pUC 19 vector (Yanisch-Perron, C. et al., Gene 33, 103-119 (1985)) in which the multiple cloning site (polylinker) is replaced by a CaMV 35 S promoter BamHI fused to a nos-tail (terminator) flanked by the 'unique' sites; EcoRI, KpnI, XhoI, and a HindIII site downstream from the promoter and EcoRI, XhoI, PstI, SphI, KpnI, HindIII upstream from the terminator. The orientation of the amorpha-4,11-diene encoding gene in pLV399 was checked by restriction analysis with PstI and NdeI. After partial digestion of this construct with KpnI the amorpha-4,11-diene encoding gene flanked by the 35S promotor and nos terminator was ligated into the KpnI digested binary vector pCGN1548.

To mobilize the recombinant binary vector to Agrobacterium tumefaciens LBA4404 (Gibco BRL, Life Technologies), a triparental mating procedure was carried out by using E.coli (DH5 α) carrying the recombinant binary vector and a helper E.coli carrying the plasmid

pRK2013 to mobilize the recombinant binary vector to A. tumefaciens LBA4404.

This transformed Agrobacterium strain was used for transformation of explants from the target plant species. Only the transformed tissue carrying a resistance marker (kanamycin-resistance, present between the binary plasmid T-DNA borders) regenerated on a selectable (kanamycin containing) regeneration medium. (According to Rogers SG, Horsch RB, Fraley RT Methods Enzymol (1986) 118: 627-640).

The plants regenerated out of the transformed tissue expressed the amorphadiene synthase gene as followed from the presence therein of amorphadiene as confirmed by GC-MS analyses.

15

EXAMPLE 6

Conversion of amorphadiene into artemisinin (DHAA) by A.annua

This assay was carried out in a way analogous to the method as described by Koepp et al. (The Journal of Biological Chemistry 270, 8686-8690 (1995)). Radioactive (³H-labeled) amorphadiene was fed to leaf discs of A.annua. For the infiltration of amorphadiene into the leaf discs of A.annua the radioactive amorphadiene can be made water soluble by complexation with cyclodextrins, for example. Radioactive amorphadiene is obtained by using the FPP-assay with the transformed E.coli BL21(DE3) cells (carrying the cloned amorphadiene synthetase gene of A.annua). Identification of the product(s) made in this assay was done by radio-GC analysis. The expected intermediates arteannuic acid (AA), dihydroarteannuic acid (DHAA) and the end product artemisinin were all used as references.

A mixture of α -cyclodextrin, β -cyclodextrin, γ -cyclodextrin, and partially ³H-labeled amorpha-4,11-diene (20 μ M) in a molar ratio of 5:5:5:1 was prepared and A.annua leaf discs were incubated in this mixture. After 120 hours of incubation artemisinic acid and

dihydroartemisinic acid could be detected by radio-GC in a way analogous to part B of example 1.

EXAMPLE 7

5 Expression of amorpha-4,11-diene synthase in transgenic A.annua and the production of artemisinin

Transformed A.annua plants were prepared as described in example 5.

For the regeneration of A.annua the medium for 10 callus, shoot and root induction consisted of Murashige and Skoog micro and macro elements including modified vitamins (Duchefa Biochemie, Haarlem, The Netherlands), 4% (w/v) sucrose, 0.1 mg/L Indole-3-acetic acid (IAA), 0.1 mg/L 6-benzylaminopurine (BAP) and 0.8% (w/v) agar 15 (Plant agar, Duchefa Biochemie, Haarlem, the Netherlands). The pH was adjusted to 5.7 with NaOH prior to the addition of agar. The medium was autoclaved at 1 bar for 20 min. Transformed explants were regenerated on this medium to fully regenerated plants.

20 The regenerated plants were found to over-express the enzyme amorpha-4,11-diene synthase which led to production of artemisinic acid, dihydroartemisinic acid, and artemisinin at a level above the natural level in non-transformed plants.

25

EXAMPLE 8

Expression of the amorpha-4,11-diene synthase gene in Saccharomyces cerevisiae and Pichia pastoris

For functional expression the cDNA clone was 30 subcloned into the inducible expression vector pYES2 (episomal vector, Invitrogen) and the constitutive expression vector (integrating the gene construct into the genome) pGAPZ A (Invitrogen). To introduce suitable restriction sites for subcloning, the gene was amplified 35 by PCR using a sense primer (primer E) 5'-CGA GAA TTC **ATG** TCA CTT ACA G-3' (introducing a EcoRI site preceding the start codon **ATG**) and an anti-sense primer (primer F) 5'-GGAT CTC GAG **TCA** TAT ACT CAT-3' (introducing a BamHI

site directly behind the stop codon **TGA**). Subcloning of the PCR product into pYES2 and pGAPZ A was done in a way analogue to Example 3.

The obtained gene constructs were transformed 5 to respectively Saccharomyces cerevisiae and Pichia pastoris using the S.cerevisiae EasyComp™ transformation kit (Invitrogen) to transform S.cerevisiae and the Pichia EasyComp™ transformation kit (Invitrogen) for transformation of P.pastoris. All transformations were 10 carried out according to the instructions of the manufacturer. Growth, selection and induction were also performed in accordance to the instructions of the manufacturer. Harvesting and sonication of the yeast cells was done in an analogous way to the method as 15 described in Example 3.

The FPP assay with the extracts of the yeast cells in which the amorpha-4,11-diene synthase gene was expressed yielded identical GC-RAGA and GC-MS chromatograms as obtained in example 4.

CLAIMS

1. Isolated DNA sequence encoding a polypeptide having the biological activity of amorpha-4,11-diene synthase.

2. DNA sequence as claimed in claim 1 which 5 exhibits at least 70% homology to the sequence as shown in Fig. 12 or the complementary strand thereof and which codes for a polypeptide having the biological activity of the enzyme amorphadiene synthase.

3. DNA sequence as claimed in claim 2, which is 10 at least 80%, preferably at least 90%, more preferably at least 95% homologous to the sequence in Fig. 12.

4. DNA sequence as claimed in claims 1-3, which has the sequence as shown in Fig. 12.

5. DNA sequence as claimed in claims 1-4, 15 characterized in that it has been isolated from plants producing amorpha-4,11-diene, for example A.annua and V.oblongifolia

6. Use of a DNA sequence as claimed in claims 1-5 for transforming or transfecting a host cell.

20 7. DNA construct comprising the DNA sequence as claimed in claims 1-5 operably linked to suitable transcription initiation and termination sequences.

8. Host cell comprising a DNA sequence as claimed in claims 1 to 5 or a DNA construct as claimed in 25 claim 7.

9. Host cell as claimed in claim 8, wherein the cell is a bacterial cell, in particular an E.coli cell.

10. Host cell as claimed in claim 8, wherein the cell is a plant cell.

30 11. Host cell as claimed in claim 10, wherein the cell is derived from a plant itself producing sesquiterpenes.

12. Host cell as claimed in claim 11, wherein the cell is an A.annua cell or a V.oblongifolia cell.

35 13. Host cell as claimed in claim 11, wherein the cell is derived from a plant selected from the group

consisting of the genera Carum, Cichorium, Daucus, Juniperus, Chamomilla, Lactuca, Pogostemon and Vetiveria.

14. Host cell as claimed in claim 10, wherein the cell is derived from a plant in which the biosynthesis of sesquiterpenoids can be induced by 5 elicitation.

15. Host cell as claimed in claim 14, wherein the cell is derived from a plant selected from the group consisting of the genera Capsicum, Gossypium, 10 Lycopersicon, Nicotiana, Phleum, Solanum and Ulmus.

16. Host cell as claimed in claim 10, wherein the cell is derived from a plant selected from the group of soybean, sunflower and rapeseed.

17. Host cell as claimed in claim 8, wherein 15 the cell is a yeast cell.

18. Host cell as claimed in claim 17, wherein the yeast cell is a Saccharomyces cerevisiae or Pichia pastoris cell.

19. Host cell as claimed in claim 17, wherein 20 the cell is an oleaginous yeast cell.

20. Host cell as claimed in claim 19, wherein the oleaginous yeast cell is a Yarrowia lipolytica cell.

21. Host cell as claimed in claims 8 and 10-16, which cell is part of a tissue or organism.

25 22. Transgenic tissue, consisting at least part of host cells as claimed in claims 8 and 10-16.

23. Transgenic organism, consisting at least part of host cells as claimed in claims 8 and 10-16.

24. Polypeptide having the biological activity 30 of the enzyme amorphadiene synthase in isolated form obtainable by isolating the polypeptide from A.annua or V. oblongifolia by a process as described in Example 1.

25. Recombinant polypeptide having the biological activity of the enzyme amorphadiene synthase 35 obtainable by expressing a DNA sequence as claimed in claims 1-5 in a suitable host cell as claimed in claims 8-20.

26. Method of preparing amorphadiene,
comprising:

- a) incubating a polypeptide as claimed in claim 24 or 25 in the presence of farnesyl pyrophosphate (FPP) 5 in an incubation medium at a suitable temperature and during a suitable period of time; and
- b) optionally isolating the amorphadiene thus formed.

27. Method of preparing amorphadiene,
10 comprising the steps of:

- a) transfecting or transforming a suitable host cell with a DNA sequence as claimed in claims 1-5 or a construct according to claim 7 to obtain transgenic host cells;
- 15 b) expressing the said DNA sequence in the presence of farnesyl pyrophosphate (FPP) to form amorphadiene; and
- c) optionally isolating the amorphadiene thus formed,

20 wherein the expression level of the amorphadiene synthase is higher in transgenic host cells, tissues or organisms harboring an endogenous version of the DNA sequence than in non-transgenic host cells, tissues or organisms.

28. Method of preparing artemisinin,
25 comprising:

- a) incubation of a polypeptide as claimed in claim 24 or 25 in the presence of farnesyl pyrophosphate (FPP) and the enzymes that further convert amorpha-4,11-diene to artemisinin in an incubation medium at a 30 suitable temperature and during a suitable period of time; and
- b) optional isolation of the artemisinin thus formed.

29. Method of preparing artemisinin,
35 comprising:

- a) transfecting or transforming a suitable host cell, tissue or organism with a DNA sequence as claimed

in claims 1-5 or a construct according to claim 7 to obtain transgenic host cells, tissues or organisms;

b) expressing the said DNA sequence in the presence of farnesyl pyrophosphate (FPP); and

5 c) optionally isolating the amorpha-4,11-diene thus formed,

wherein the transgenic host cells, tissues or organisms harbor the genetic information coding for the enzymes that further convert amorpha-4,11-diene to artemisinin

10 and wherein the expression level of the amorpha-4,11-diene synthase is higher in transgenic host cells,

tissues or organisms harboring an endogenous version of the DNA sequence than in non-transgenic host cells, tissues or organisms.

15 30. Source of artemisinin, comprising host cells, tissues or organisms harboring a DNA sequence as claimed in claims 1-5 and the genetic information coding for the enzymes that further convert amorpha-4,11-diene to artemisinin, which host cells, tissues or organisms 20 have expressed the said DNA sequence.

31. Source as claimed in claim 30, wherein the cells are bacterial cells, yeast cells or plant cells.

32. Source as claimed in claim 30, wherein the cells are disrupted.

25 33. Transgenic cell, tissue or organism harboring in its genome more copies of a DNA sequence as claimed in claims 1-5 than are present in a corresponding non-transgenic cell, tissue or organism.

34. Transgenic cell as claimed in claim 33, 30 which cell is an E.coli cell.

35. Transgenic cell as claimed in claim 33, which cell is a Saccharomyces cerevisiae cell.

36. Transgenic cell as claimed in claim 33, which cell is an oleaginous cell, in particular a Yarrowia lipolytica cell.

37. Transgenic organism as claimed in claim 33, which organism is a plant itself producing sesquiterpenes.

38. Transgenic organism as claimed in claim 37,
which organism is A.annua or V.oblongifolia.

39. Transgenic organism as claimed in claim 37,
which organism is a plant selected from the group
5 consisting of the genera Carum, Cichorium, Daucus,
Juniperus, Chamomilla, Lactuca, Pogostemon and Vetiveria.

40. Transgenic organism as claimed in claim 33,
which organism is a plant in which the biosynthesis of
sesquiterpenoids can be induced by elicitation.

10 41. Transgenic organism as claimed in claim 40,
which organism is a plant selected from the group
consisting of the genera Capsicum, Gossypium,
Lycopersicon, Nicotiana, Phleum, Solanum and Ulmus.

42. Transgenic organism as claimed in claim 33,
15 which organism is a plant selected from the group
consisting of soybean, sunflower and rapeseed.

09/763822

PCT/EP99/06302

WO 00/12725

1/18

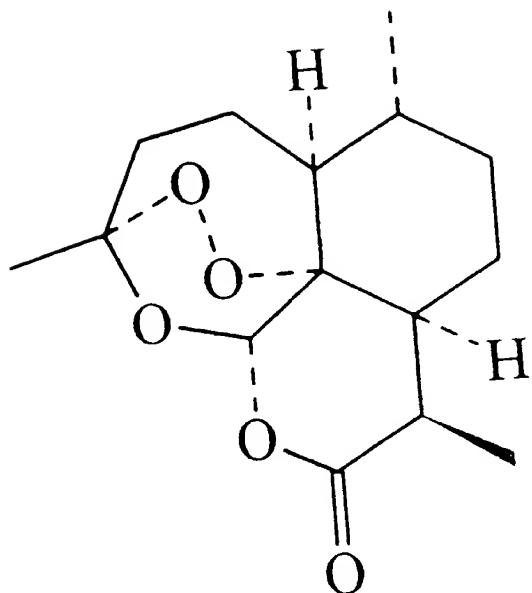


FIG. 1

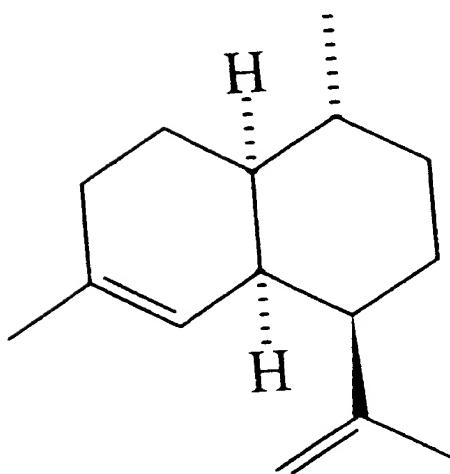


FIG. 4

09/763822

PCT/EP99/06302

WO 00/12725

2/18

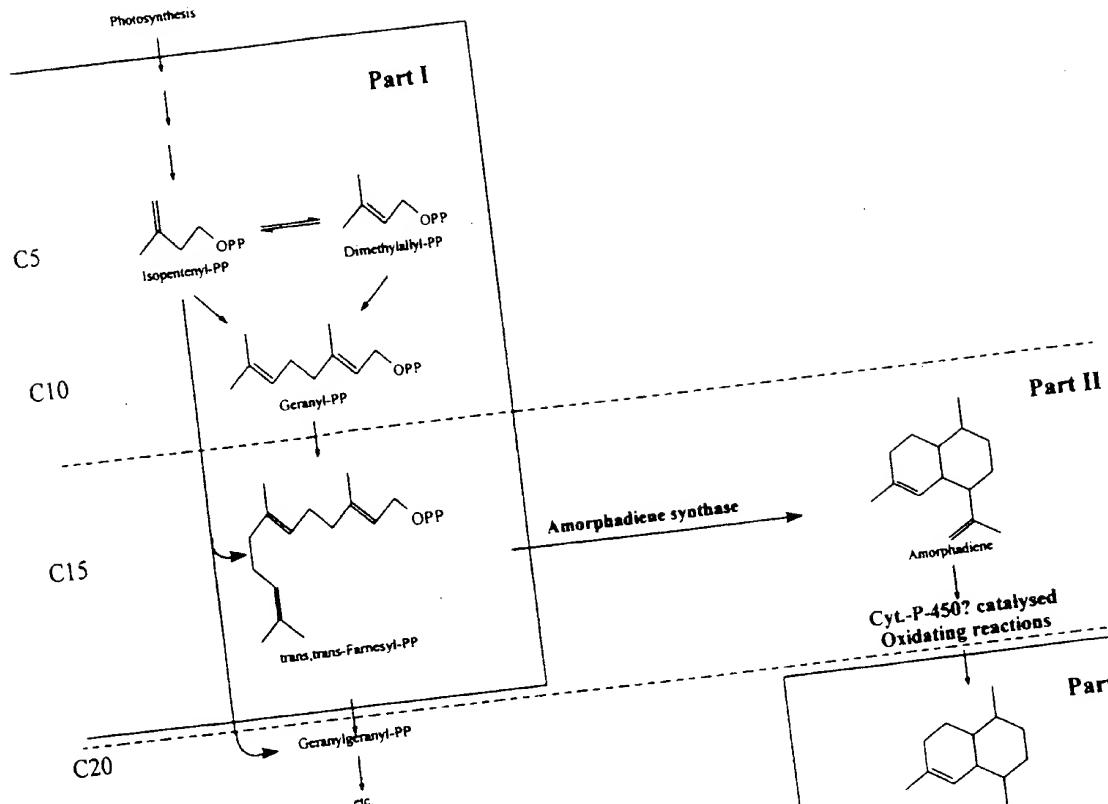
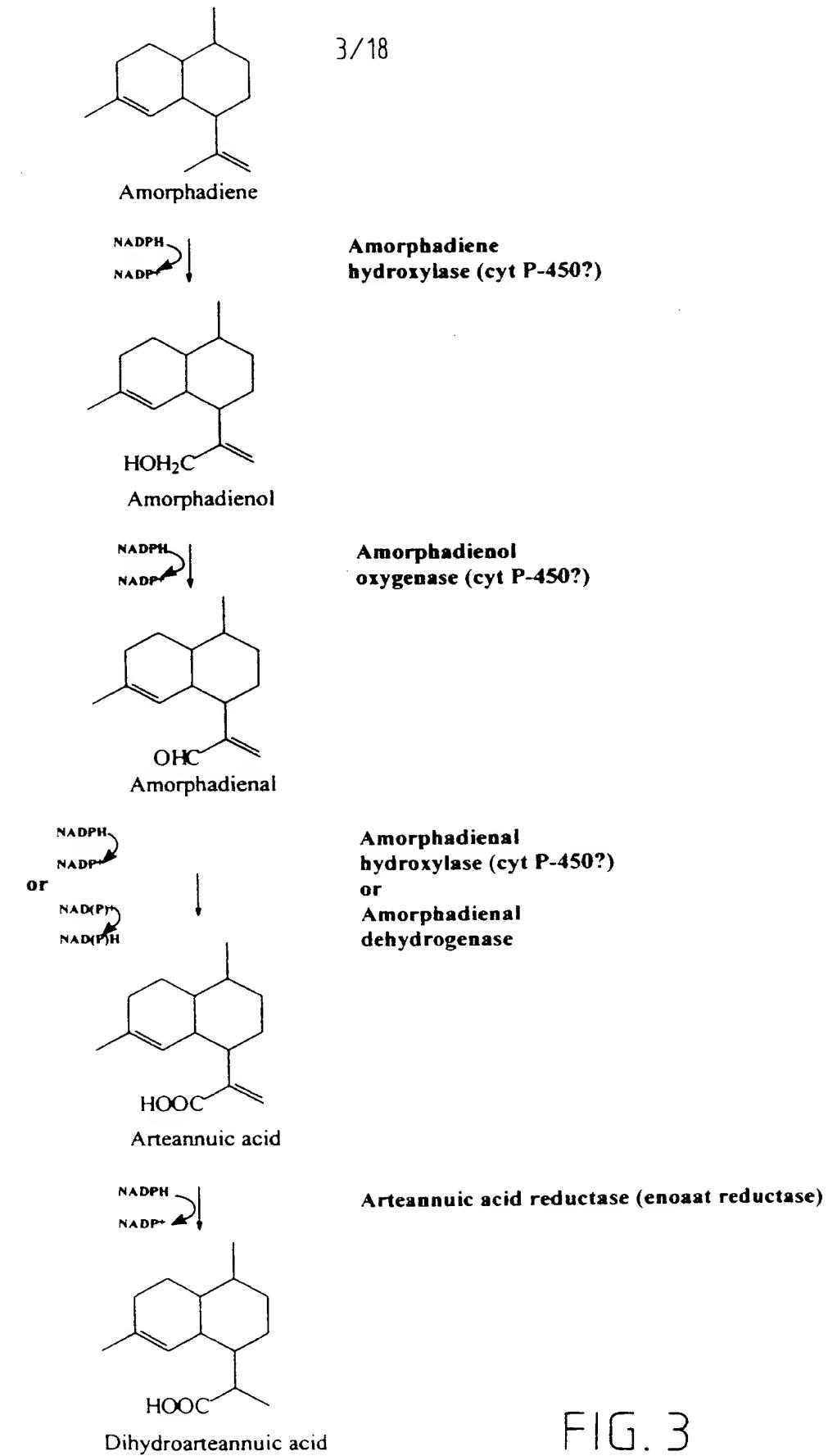


FIG. 2

3/18

FIG. 3

4/18

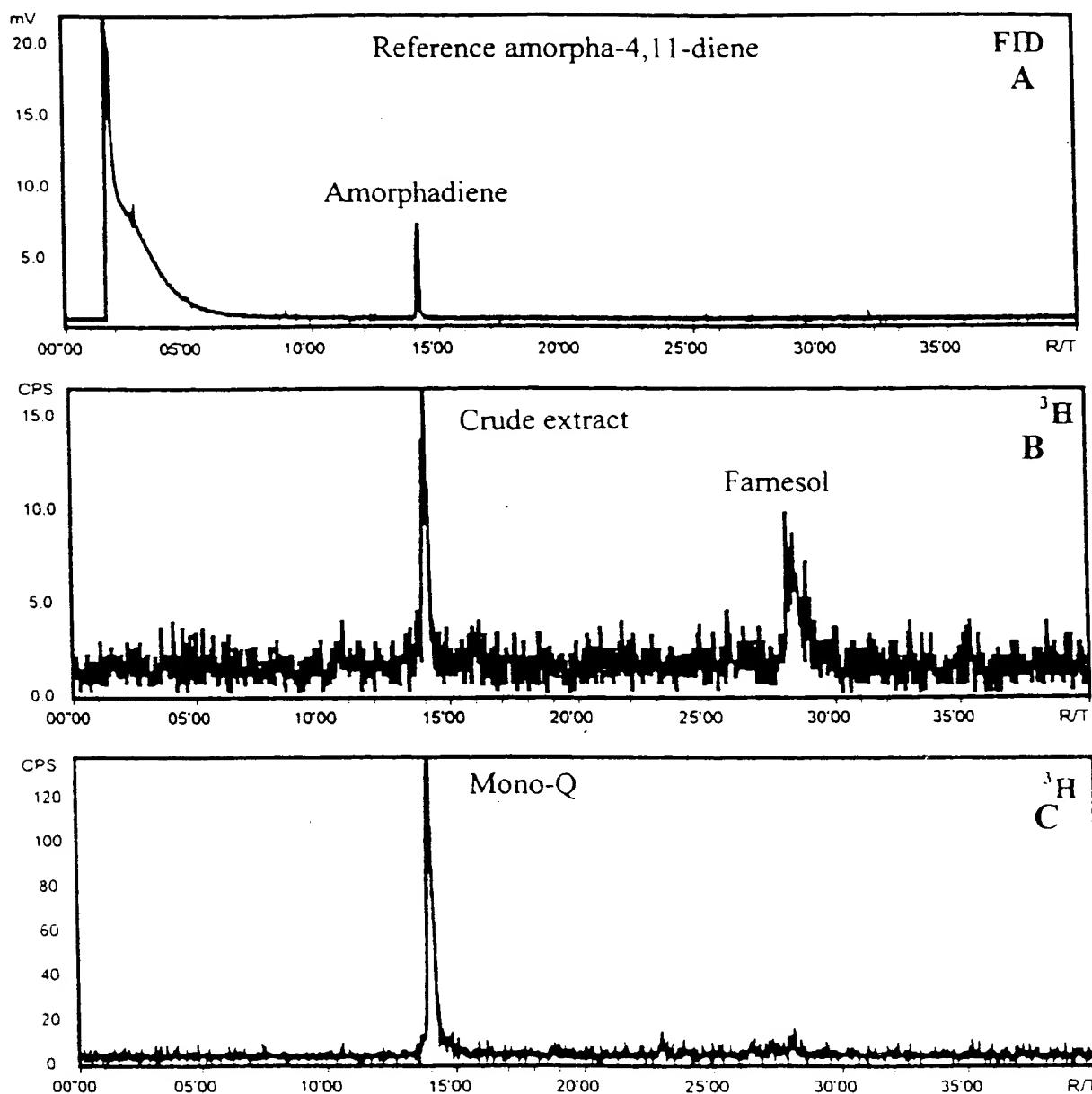


FIG. 5

5/18

Library Searched : C:\DATABASE\WITLOF.L
Quality : 99
ID : amorpha-4,11-dieen

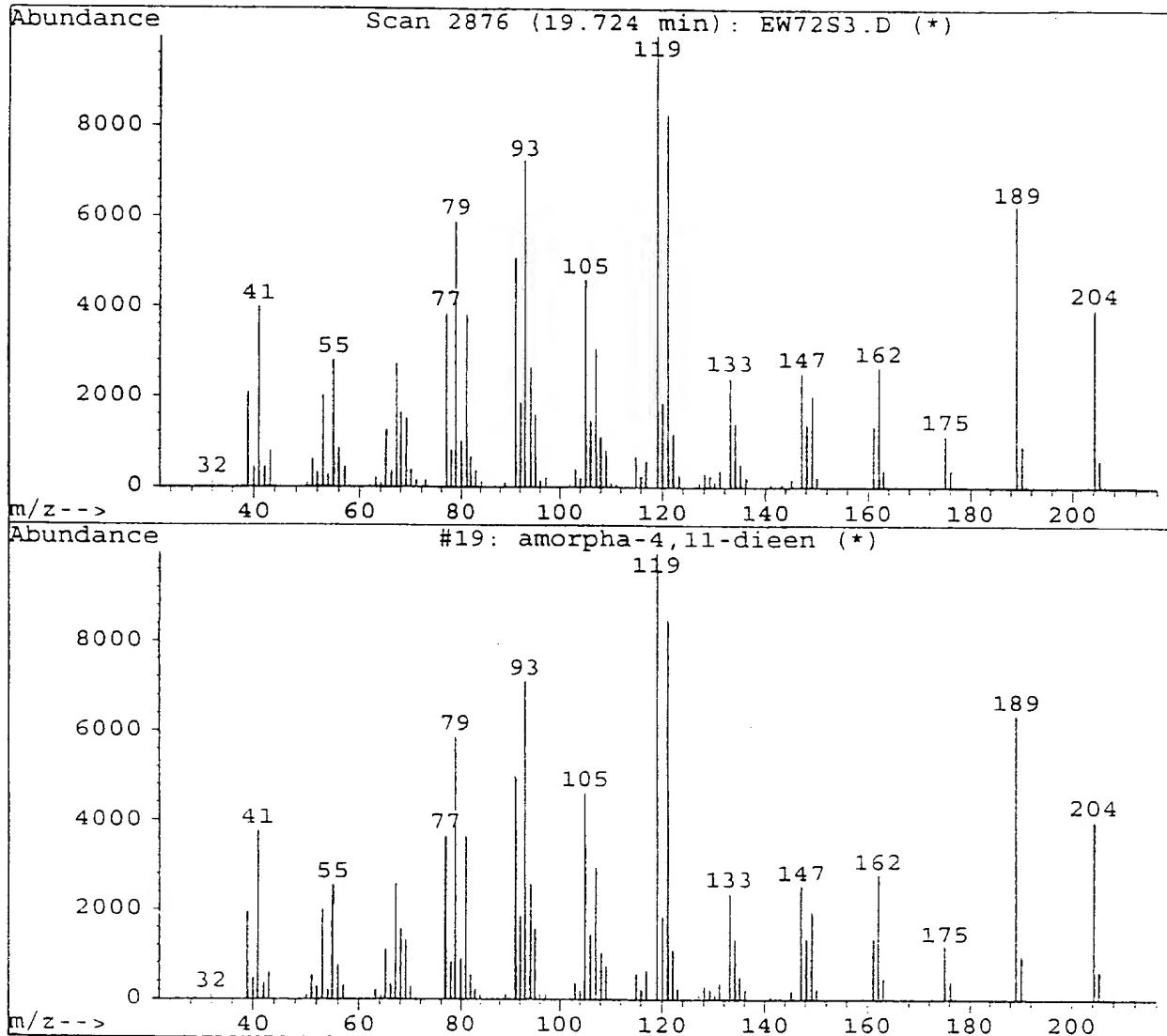


FIG. 6

6/18

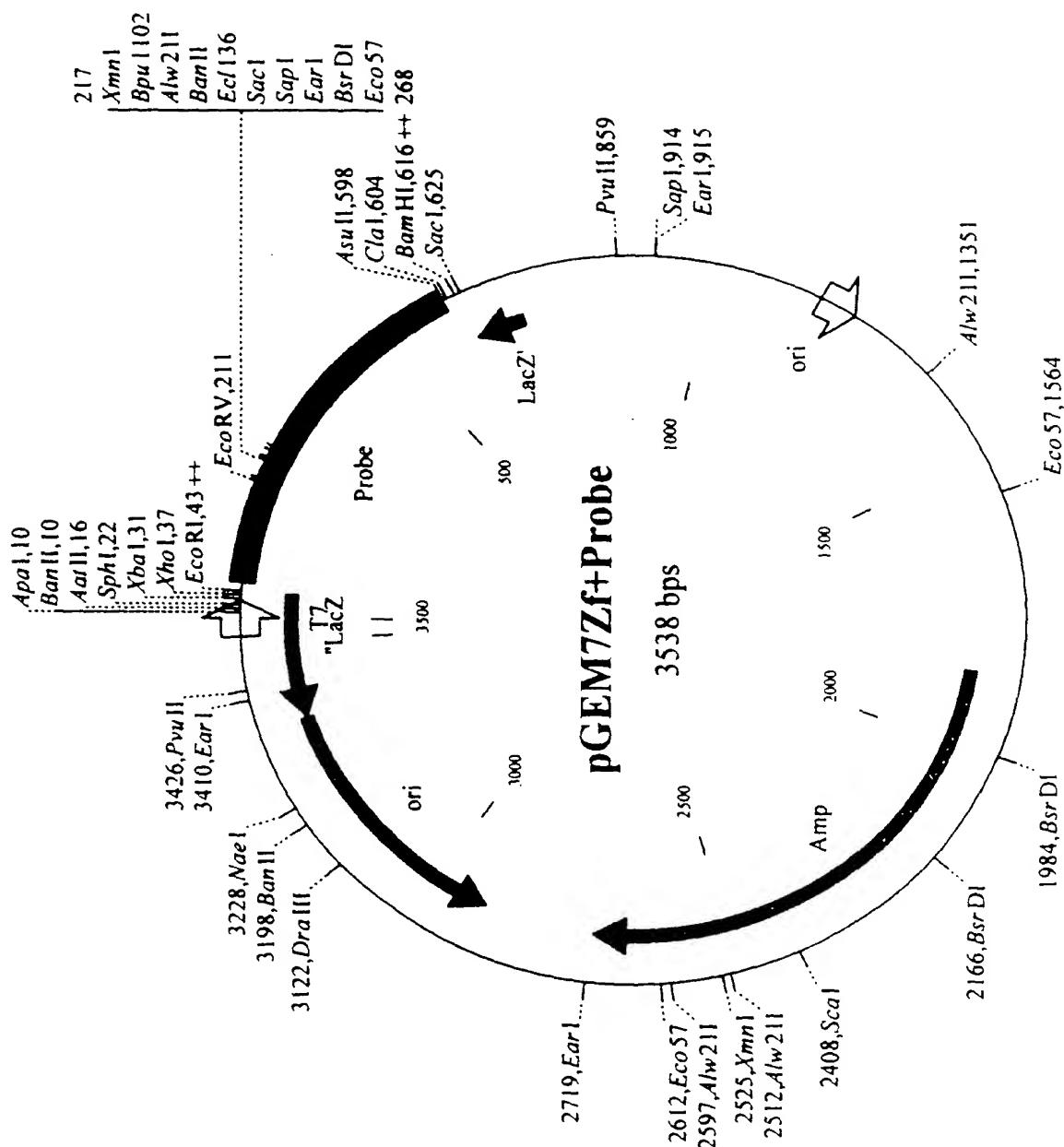


FIG. 7

7/18

27 gat gag aat ggg aaa ttt aag gaa tcg tta gct aat gat gtt gaa ggt ttg
 D E N G K F K E S L A N D V E G L

78 ctt gag ttg tac gaa gca act tct atg agg gta cct ggg gag att ata tta
 L E L Y E A T S M R V P G E I I L

129 gaa gat gct ctt ggt ttt aca cga tct cgt ctt agc att atg aca aaa gat
 E D A L G F T R S R L S I M T K D

180 gct ttg tct aca aac ccc gct ctt ttt acc ctt gaa ata caa cgg gca cta aag
 A F S T N P A L F T E I Q R A L K

231 caa ccc ctt tgg aaa agg ttg cca aga ata gag gcg gcg cag tac att cct
 Q P L W K R L P R I E A Q Y I P

282 ttc tat caa caa caa gat tct cat aac aag act tta ctt aaa ctt gct aag
 F Y Q Q D S H N K T L L K L A K

333 tta gag ttc aat ttg ctt cag tca ttg cac aag gaa gag ctc agc cat gtg
 L E F N L L Q S L H K E E L S H V

384 tgc aaa tgg tgg aaa gct ttc gat atc aag aac gca cct tgt tta aga
 C K W K A F D I K K N A P C L R

435 gat aga att gtt gaa tgc tac ttt tgg gga cta ggt tca ggc tat gag cca
 D R I V E C Y F W G L G S G Y E P

486 cag tat tcc cgg gct aga gtt ttc ttc aca aaa gct gtt gtt ata act
 Q Y S R A R V F F T K A V A V I T

537 ctt ata gac gac acc ttc gac gct acg g
 L I D D T F D A T

Primer A

Primer B

FIG. 8

8/18

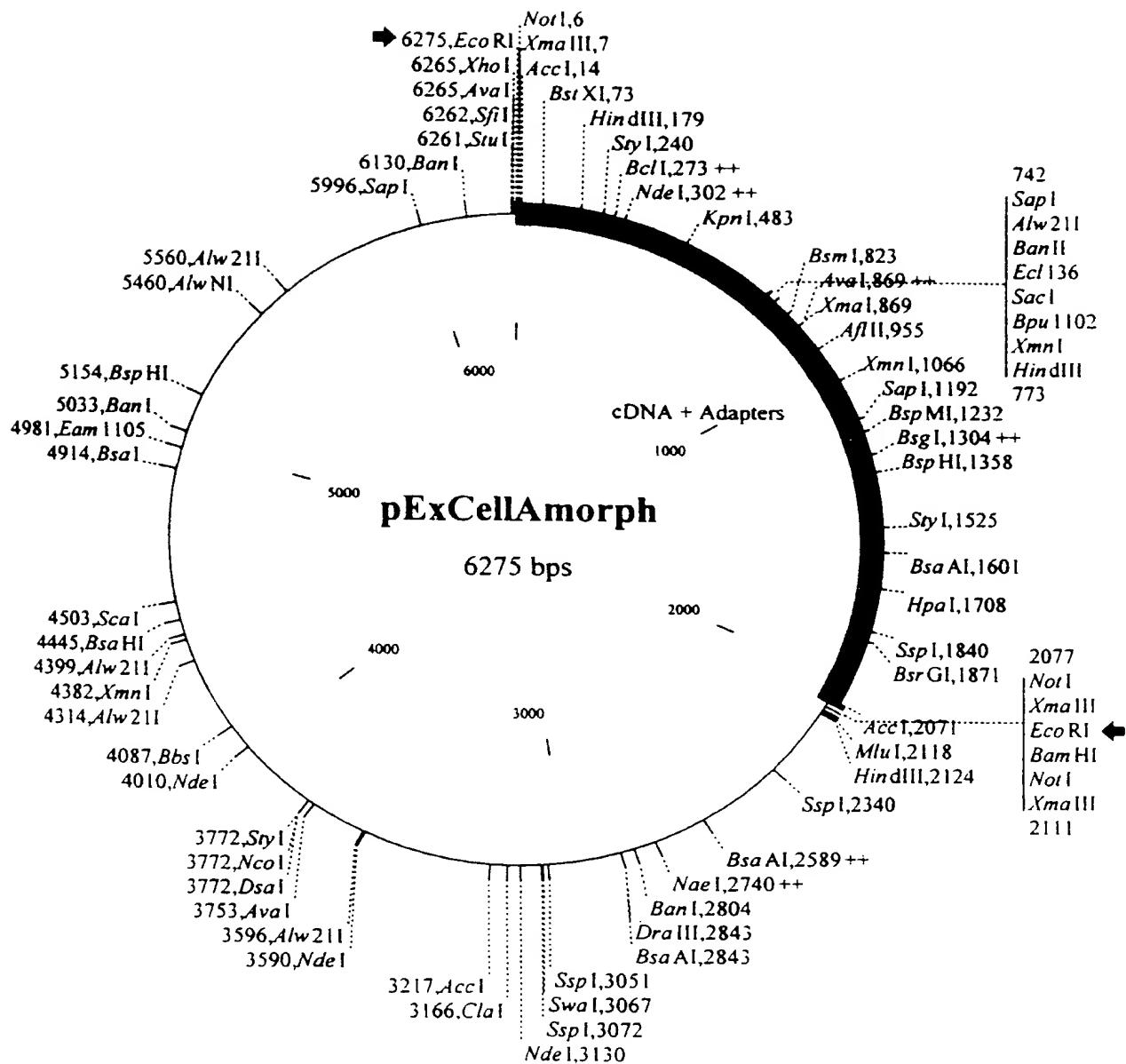


FIG. 9

EcoR I (Not I) Adapter

1 aattcgcggc cgctcgaca aatcatgtca cttacagaag aaaaacctat
 N S R P R R Q I M S L T E E K P
 ← *EcoR I* *Not I*

51 tcgccccatt gccaactttc ctccaagcat ttggggagat cagttctca
 I R P I A N F P P S I W G D Q F L

101 tctatcaaaa gcaagtagag caaggggtgg aacagatagt gaatgattta
 I Y Q K Q V E Q G V E Q I V N D L

151 aaaaaagaag tgcggcaact actaaaagaa gctttggata ttccttatgaa
 K K E V R Q L L K E A L D I P M

201 acatgccaat ttgttgaagc tgattgatga aattcaacgc cttggaatac
 K H A N L L K L I D E I Q R L G I

251 cgtatcactt tgaacggag attgatcatg cattgcaatg tatttatgaa
 P Y H F E R E I D H A L Q C I Y E

301 acatatggtg ataactggaa tggtgaccgc tcttccttat ggttccgtct
 T Y G D N W N G D R S S L W F R

351 tatgcgaaag caaggatatt atgttacatg tcatgtttc aataactata
 L M R K Q G Y Y V T C D V F N N Y

401 aagacaaaaaa tggagcgttc aagcaatcgt tagctaata gttgaaggt
 K D K N G A F K Q S L A N D V E G

451 ttgcttgagt tgtacgaagc aacttctatg agggtacctg gggagattat
 L L E L Y E A T S M R V P G E I

501 attagaagat gctcttggtt ttacacgatc tcgtcttagc attatgacaa
 I L E D A L G F T R S R L S I M T

551 aagatgctt ttctacaaac cccgctcttt ttaccgaaat acaacggca
 K D A F S T N P A L F T E I Q R A

601 ctaaagcaac ccctttggaa aaggttgcca agaatagagg cggcgcatg
 L K Q P L W K R L P R I E A A Q

651 cattcccttc tatcaacaac aagattctca taacaagact ttacttaaac
 Y I P F Y Q Q Q D S H N K T L L K

701 ttgctaagtt agagttcaat ttgcttcagt cattgcacaa ggaagagctc
 L A K L E F N L L Q S L H K E E L

751 agccatgtgt gcaaataggtg gaaagcttgc gatatacaaga agaacgcacc
 S H V C K W W K A F D I K K N A

801 ttgtttaaga gatagaattt gttgaatgcta cttttggga ctaggttcag
 P C L R D R I V E C Y F W G I G S

851 gctatgagcc acagtattcc cgggcttagag ttttcttcac aaaagctgtt
 G Y E P O Y S R A R V F F T K A V

FIG. 10-1

10/18

901 gctgttataa ctcttataga tgacacttat gatgcgtatg gtacttatga
 A V I T L I D D T Y D A Y G T Y
 951 agaacttaag atcttactg aagctgtga aaggtggta attacatgct
 E E L K I F T E A V E R W S I T C
 1001 tagacacact tccagaatac atgaaaccga tatacaaatt attcatggat
 L D T L P E Y M K P I Y K L F M D
 1051 acatacacag aatggaaga atttcttgca aaggagggaa gaacagatct
 T Y T E M E F L A K E G R T D
 1101 atttaactgc ggcaaagaat ttgtgaaaga gtttgttata aacctgatgg
 L F N C G K E F V K E F V R N L M
 1151 ttgaagcaaa atgggcaa at gaggacaca taccaaccac tgaagagcat
 V E A K W A N E G H I P T T E E H
 1201 gatccagttg taatcattac tggcggtgct aacctgctta caacaacttg
 D P V V I I T G G A N L L T T T
 1251 ttatcttggc atgagtgata tattcacaaa agagtctgatc gaatggctg
 C Y L G M S D I F T K E S V E W A
 1301 tctctgcacc tcctctttt agataactcag gtataacttgg tcgacgccta
 V S A P P L F R Y S G I L G R R L
 1351 aatgatctca tgaccacaa ggccgagcaa gaaagaaaac atagttcatc
 N D L M T H K A E Q E R K H S S
 1401 gaggcttggaa agttatataa aggaatataa tgtcaatgag gagtatgccc
 S S L E S Y M K E Y N V N E E Y A
 1451 aaaccttgc ttacaaggaa gtagaaagatg tgtggaaaga tataaaccga
 Q T L I Y K E V E D V W K D I N R
 1501 gagtacctca caactaaaaa cattccaagg ccgttattga tggctgtat
 E Y L T T K N I P R P L L M A V
 1551 ctatgtgc cagttcttg aagttcaata tgcagggaaag gataacttca
 I Y L C Q F L E V Q Y A G K D N F
 1601 cacgtatggg agacgaatac aaacatctca taaagtctct actcgttat
 T R M G D E Y K H L I K S L L V Y
 1651 cctatgatgta tatgactacc aatccttcgt gcatagccta tcaatttat
 P M S I - L P I L R A - P I N Y
 1701 tgaagggtt aactatgcac gtctctatgg agagaatttc tcaagctatt
 I E R V N Y A R L Y G E N F S S Y

FIG. 10-2

11/18

1751 tggtgtttct tgctggcaat aataaatcag acgcataaaa ttgtattgaa
 L V F L A G N N K S D A - N C I E
 1801 cstatatgccg atagctattt aaagttatta tacaactaaa atattcaaca
 L Y A D S Y L K L L Y N - N I Q
 1851 atggtattat acttttactt tgtacaaaag caaaagtaca ctactgttat
 Q W Y Y T F T L Y K S K S T L L L
 1901 gtaacatttt agttctatga tactttagtt acgaatcggc ttatatacat
 C N I L V L - Y F S Y E S A Y I H
 1951 tgatacactt ttatgcagaa aaccctagta aataaaaagt cgatatcttg
 - Y T F M Q K T L V N K K S I S
 2001 tactacacat atcgacacgaa tttccgttg ccgttgtat tttacgatat
 C T T H I A R I S V C R L Y F T I
 2051 gttatttaat gaatatgttt catgtggttg ttgctaaaa aaaaagtgcga
 C Y L M N M F H V V V A - K K S R
 [*Not* I] [*Eco* RI]
 2101 cgcggccgcg aa
 R G R] E
Eco RI (*Not* I) Adapter

FIG. 10-3

12/18

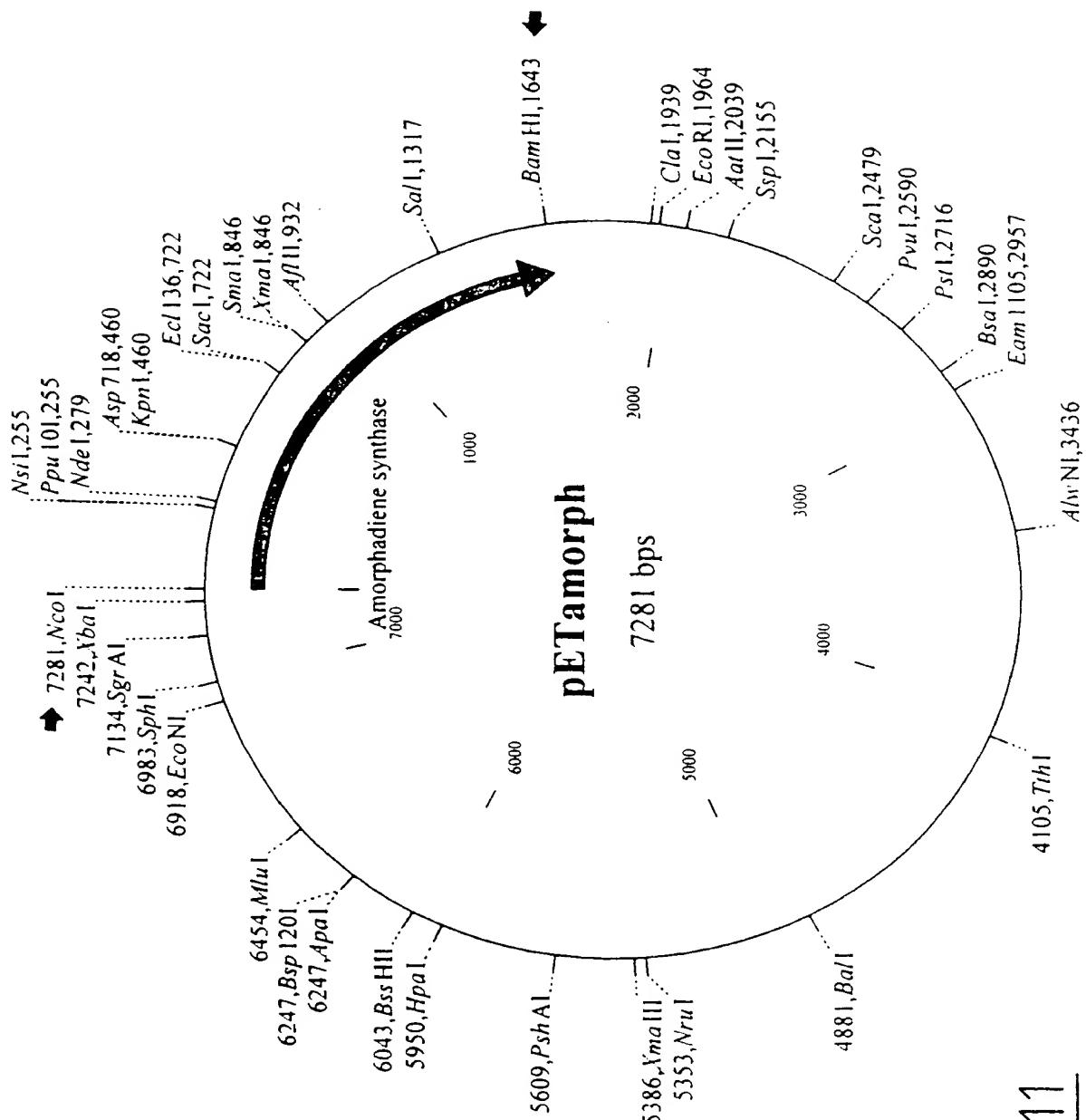


FIG. 11

7281 $\lceil NcoI \rceil$ 13/18
 7281 ccatggcact tacagaagaa aaacctattc gccccattgc caactttcct
 T M A L T E E K P I R P I A N F P
 Start codon
 50 ccaagcattt ggggagatca gtttctcatc tatcaaaagc aagtagagca
 P S I W G D Q F L I Y Q K Q V E
 100 aggggtggaa cagatagtga atgattaaa aaaagaagtg cggcaactac
 Q G V E Q I V N D L K K E V R Q L
 150 taaaagaagc tttggatatt cctatgaaac atgccaattt gttgaagctg
 L K E A L D I P M K H A N L L K L
 200 attgatgaaa ttcaacgcct tggatatccg tatcactttg aacgggagat
 I D E I Q R L G I P Y H F E R E
 250 tgatcatgca ttgcaatgt a ttatgaaac atatggat aactggaatg
 I D H A L Q C I Y E T Y G D N W N
 300 gtgaccgctc ttccttatgg ttccgtctta tgcgaaagca aggatattat
 G D R S S L W F R L M R K Q G Y Y
 350 gttacatgtg atgtttcaa taactataaa gacaaaaatg gagcgttcaa
 V T C D V F N N Y K D K N G A F
 400 gcaatcgta gctaattatg ttgaaggttt gcttgagttg tacgaagcaa
 K Q S L A N D V E G L L E L Y E A
 450 cttctatgag ggtacctggg gagattata tagaagatgc tcttggttt
 T S M R V P G E I I L E D A L G F
 500 acacgatctc gtcttagcat tatgacaaaa gatgctttt ctacaaaccc
 T R S R L S I M T K D A F S T N
 550 cgcttttt accgaaatac aacggcact aaagcaaccc ctttggaaaa
 P A L F T E I Q R A L K Q P L W K
 600 gttgccaag aatagaggcg ggcgcgtaca ttcccttcta tcaacaacaa
 R L P R I E A A Q Y I P F Y Q Q Q
 650 gattctata acaagacttt acttaaactt gctaagttt agttcaattt
 D S H N K T L L K L A K L E F N
 700 gttcagtca ttgcacaagg aagagctcg ccatgtgtgc aaatgggaa
 L L Q S L H K E E L S H V C K W W
 750 aagcttcga tatcaagaag aacgcacctt gtttaagaga tagaatttt
 K A F D I K K N A P C L R D R I V
 800 gaatgctact tttggggact agttcaggg tatgagccac agtattcccg
 E C Y F W G L G S G Y E P Q Y S
 850 ggctagagtt ttcttcacaa aagctgtgc tggataact cttatagatg
 R A R V F F T K A V A V I T L I D

FIG. 12-1

09/763822

WO 00/12725

PCT/EP99/06302

14/18

900 acacttatga tcgcgtatgg acttatgaag aacttaagat ctttactgaa
 D T Y D A Y G T Y E E L K I F T E
 950 gctgttgaaa ggtggtaat tacatgccta gacacacttc cagaatacat
 A V E R W S I T C L D T L P E Y
 1000 gaaaccgata tacaaattat tcatggatac atacacagaa atggaagaat
 M K P I Y K L F M D T Y T E M E E
 1050 ttcttgcaaa ggagggaga acagatctat ttaactgcgg caaagaattt
 F L A K E G R T D L F N C G K E F
 1100 gtgaaaagagt ttgttagaaa cctgatggtt gaagcaaaat gggcaaata
 V K E F V R N L M V E A K W A N
 1150 gggacacata ccaaccactg aagagcatga tccagttgta atcattactg
 E G H I P T T E E H D P V V I I T
 1200 gcggtgctaa cctgcttaca acaacttggtt atcttggcat gagtgatata
 G G A N L L T T T C Y L G M S D I
 1250 ttcacaaaaag agtctgtcga atgggctgtc tctgcaccc tccttttag
 F T K E S V E W A V S A P P L F
 1300 atactcaggat atacttggtc gacgcctaaa tgcatttcatt acccacaagg
 R Y S G I L G R R L N D L M T H K
 1350 ccgagcaaga aagaaaacat agttcatcga gccttggaaat ttatataa
 A E Q E R K H S S S S L E S Y M K
 1400 gaatataatg tcaatgagga gtatgccaa accttgattt acaaggaagt
 E Y N V N E E Y A Q T L I Y K E
 1450 agaagatgtg tggaaagata taaaccgaga gtacccatcaca actaaaaaca
 V E D V W K D I N R E Y L T T K N
 1500 ttccaaggcc gttattgtatg gctgtgatct atttgccttgc gtttcttggaa
 I P R P L L M A V I Y L C Q F L E
 1550 gttcaatatg cagggaaagga taacttcaca cgtatgggag acgaatacaa
 V Q Y A G K D N F T R M G D E Y
 1600 acatctcata aagtctctac tcgtttatcc tatgagtata tgaggatcc
 K H L I K S L L V Y P M S I - G S
 BamHI
 Stop codon

FIG. 12-2

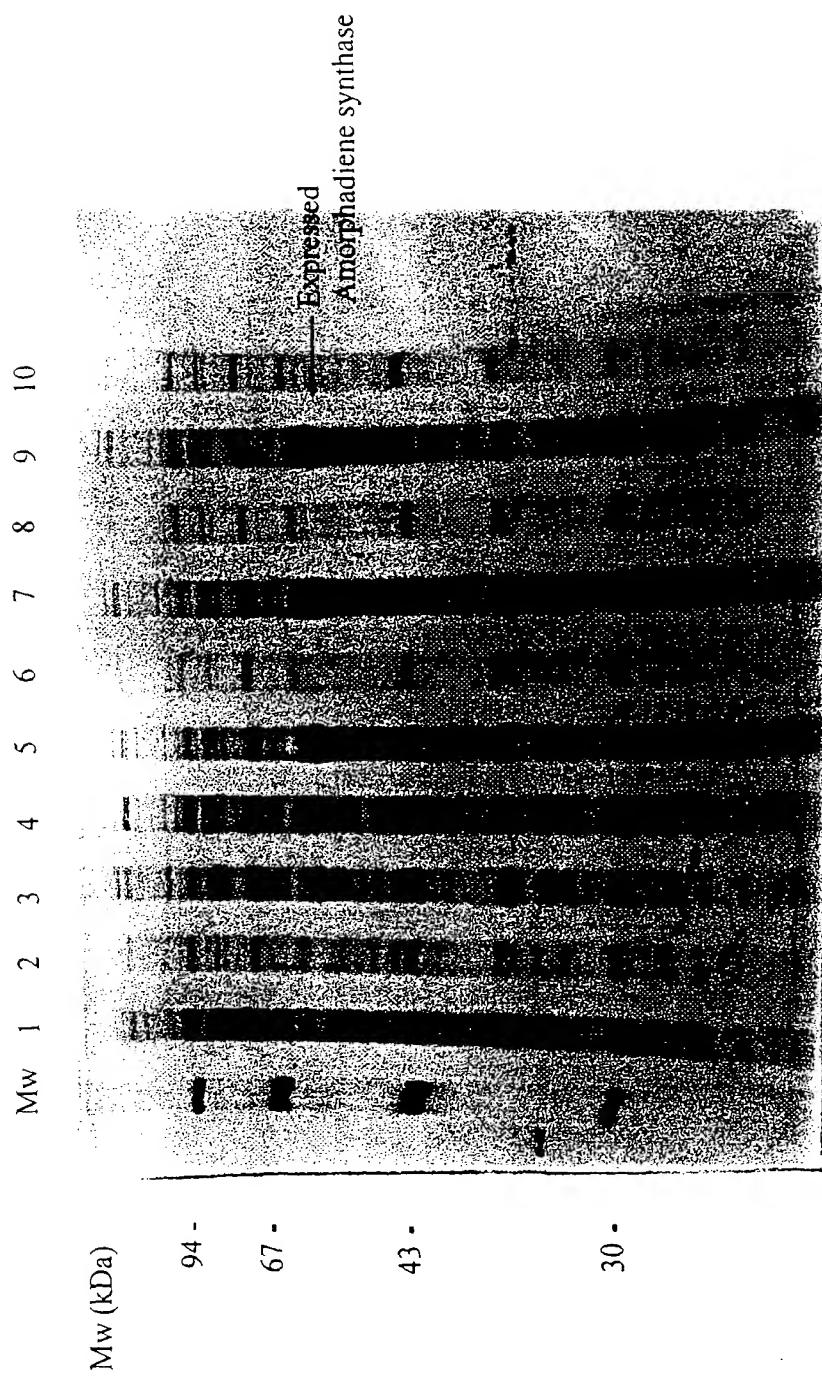


FIG. 13

09/763822

WO 00/12725

PCT/EP99/06302

16/18

WITLOF-EH2751B

monster 1 + amorph, farnesol

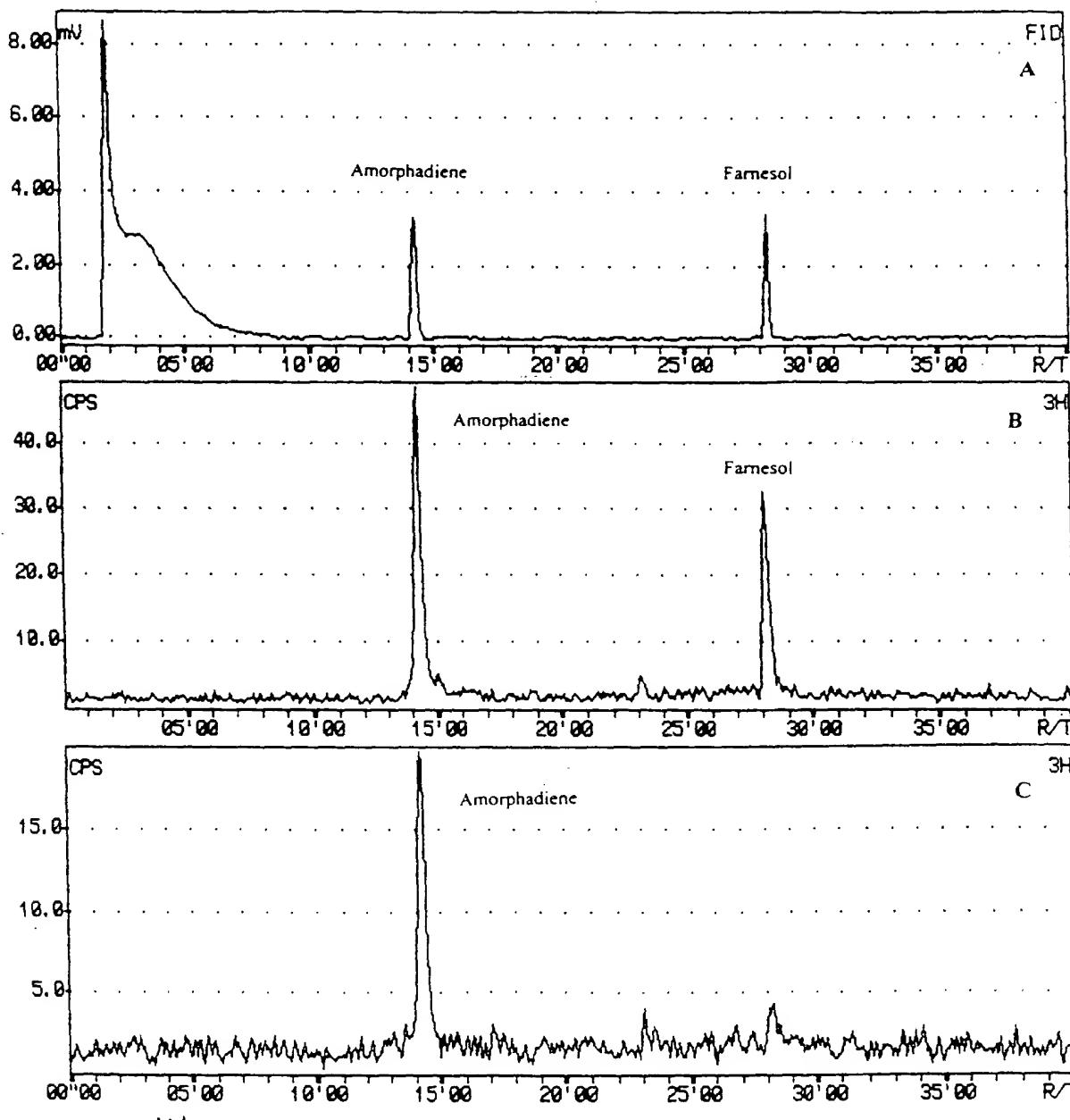
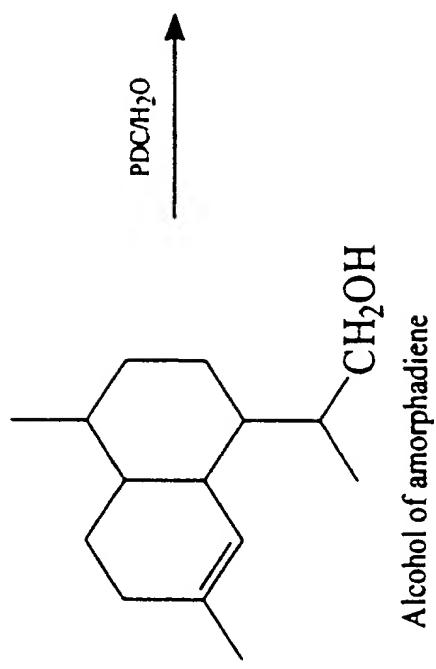
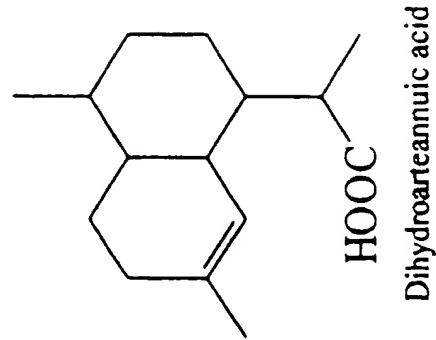


FIG. 14



1.) Sterically hindered
 BH_3 (also enatio-
selective)
2.) $\text{NaOH}/\text{H}_2\text{O}_1$

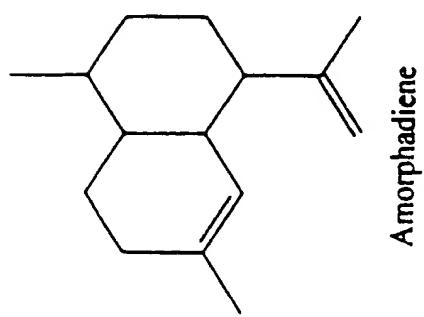
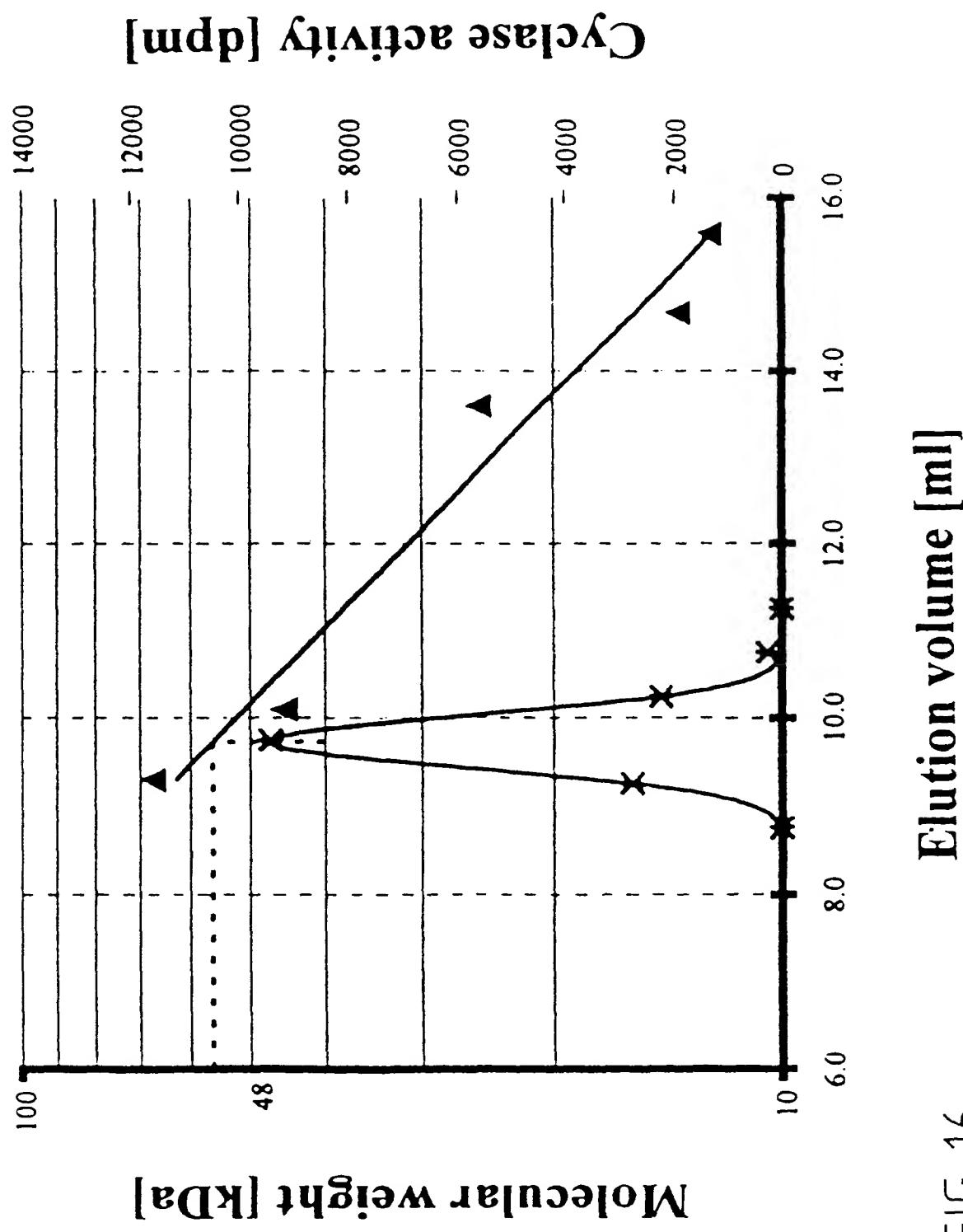


FIG. 15

18/18



INTERNATIONAL SEARCH REPORT

Final Application No

PCT/EP 99/06302

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	C12N15/60	C12N15/70	C12N15/82	C12N9/88	C12N5/10
	C12N1/19	C12N1/21	C12P5/00	C12P17/18	A01H5/00
//C12R1:19, C12R1:84, C12R1:865					

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRODELIUS P. ET AL.: "Metabolic engineering of plant secondary metabolism: a tool to improve the productivity of plant cell cultures?" ABSTRACT PAPERS OF THE AMERICAN CHEMICAL SOCIETY, 213 MEETING, April 1997 (1997-04), page AGFD026 XP002091772 abstract	1-42
A	WOERDENBAG H J ET AL: "Progress in the research of artemisinin -related antimalarials: an update." PHARMACY WORLD AND SCIENCE, (1994 AUG 5) 16 (4) 169-80. REF: 157 JOURNAL CODE: B07. ISSN: 0928-1231., XP002091773 Netherlands the whole document	1-42 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the International filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the International filing date but later than the priority date claimed

- "T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the International search

Date of mailing of the International search report

11 February 2000

28/02/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 851 epo nl.
 Fax: (+31-70) 340-3016

Authorized officer

Kania, T

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/06302

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VAN GELDRE E. ET AL.: "State of the art of the production of the antimalarial compound artemisinin in plants" PLANT MOLECULAR BIOLOGY, vol. 33, no. 2, 1997, pages 199-209, XP002091774 the whole document	1-42
A	WALLAART T. ET AL.: "Bioconversion of dihydroartemisinic acid into the new antimalarial drug artemisinin" PHARMACY WORLD AND SCIENCE, vol. 16, no. 3, 1994, page C4 XP002091775 abstract	1-42
A	VERGAUWE A. ET AL.: "Agrobacterium tumefaciens-mediated transformation of <i>Artemisia annua</i> L. and regeneration of transgenic plants" PLANT CELL REPORTS, vol. 15, no. 12, 1996, pages 929-933, XP002091776 the whole document	1-42
A	WO 94 00584 A (WORCESTER POLYTECH INST) 6 January 1994 (1994-01-06) the whole document	1-42
A	MATSUSHITA Y ET AL: "Cloning and analysis of a cDNA encoding farnesyl diphosphate synthase from <i>Artemisia annua</i> " GENE, NL, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 172, no. 2, 26 June 1996 (1996-06-26), pages 207-209, XP004042738 ISSN: 0378-1119 the whole document	1-42
A	BRODELIUS, PETER E.: "Metabolic engineering of secondary metabolism in <i>vanilla planifolia</i> and <i>artemisia annua</i> ." BOOK OF ABSTRACTS, 211TH ACS NATIONAL MEETING, NEW ORLEANS, LA, MARCH 24-28 (1996), BIOT-002 PUBLISHER: AMERICAN CHEMICAL SOCIETY, WASHINGTON, D. C., XP002130362 abstract	1-42
	-/-	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/06302

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PARK C. ET AL.: "Expression, secretion, and processing of rice alpha-amylase in the yeast <i>Yarrowia lipolytica</i>" <i>JOURNAL OF BIOLOGICAL CHEMISTRY</i>, vol. 272, no. 11, 1997, pages 6876-6881, XP002130363 cited in the application the whole document</p> <p>—</p>	17-20, 31,35,36
A	<p>CHANG C. ET AL.: "Improvement of heterologous protein productivity using recombinant <i>Yarrowia lipolytica</i> and cyclic fed-batch process strategy" <i>BIOTECHNOLOGY AND BIOENGINEERING</i>, vol. 59, no. 3, 5 August 1998 (1998-08-05), pages 379-385, XP002130364 the whole document</p> <p>—</p>	17-20, 31,35,36

INTERNATIONAL SEARCH REPORT

Information on patent family members

Final Application No

PCT/EP 99/06302

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9400584	A 06-01-1994	NONE	